PR. J.M. BOUQUEGNEAU
Faculty of Sciences
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TRACE METAL CONTAMINATION AND DETOXICATION PROCESSES IN MARINE MAMMALS FROM EUROPEAN COASTS

KRISHNA DAS

Dissertation presented in fulfillment for the degree of Doctor of Sciences (Section Oceanology)

UNIVERSITÉ de Liège

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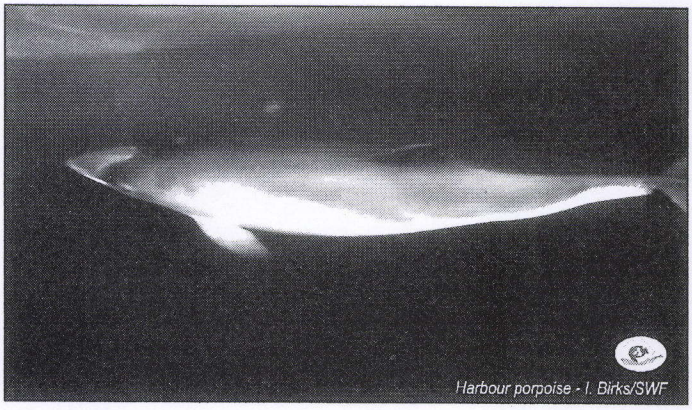

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"One can measure the greatness and the moral progress of a nation by looking at how it treats her animals"

Mahatma Ghandi

Remerciements

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FOREWORD

Marine mammals are key elements in our marine ecosystems. As K-selected species, they are likely to have a strong effect in structuring their environment. As top-predators, they are potential indicators of ecosystem health (Bouquegneau et al., 1997; Bowen, 1997; Reijnders and Aguilar, 2002).

Awareness of the threat of environmental contaminants to marine mammals is widespread. High concentrations of certain compounds in the tissues of these animals have in the past been associated with organ anomaly, impaired reproduction and immune function, as shown by large die-offs among seal and cetacean species. Indeed, recent mass-mortalities and stock declining among several marine mammal populations from highly polluted areas have among others been attributed to the contamination by organochlorines such as PCBs (Reijnders and Aguilar, 2002). Several investigations have been carried out in an attempt to evaluate organic contaminant effects at ambient environmental levels (Reijnders, 1986; Aguilar and Borrel, 1994; De Guise *et al.,* 1995; de Swart *et al.,* 1994). It has been demonstrated that seals fed polluted fish from Dutch Wadden Sea (Southern North Sea) showed reduced pup production when compared to those fed much less polluted fish from the Northeast Atlantic (Reijnders *et al.,* 1986). This study was the first sign of a causal relationship between naturally occurring levels of pollutants and a physiological response in marine mammals.

However, a clear cause and effect relationship between residue levels of organic contaminants and the observed effects has been demonstrated in a few studies only. Assessing the impact of other contaminants such as trace metals on marine mammal populations is even more difficult as metals are not strictly man-made chemicals. They are driven to the oceans by rivers, dumping discharges, atmospheric inputs and can be found naturally in all compartments of the biosphere (Clark, 2001).

The main reason for the lack of proof of the impact of pollution on marine mammals is the difficulty or impossibility of experimenting in laboratory conditions with these animals and the frequent occurrence of confounding factors that hamper the establishment of a direct causal relationship. Examples of these factors are the fact that pollution always occurs in a mixture of a large number of chemical compounds, the lack of data on biological variables influencing tissue levels, quality of samples analysed, the limited information on pathology and occurrence of

diseases in the studied specimen, the absence of reliable population data, and the lack of information on the influence of other detrimental factors, such as the impact of fisheries and other human-related sources of disturbance.

One approach to this problem is to carry out systematic post-mortem investigations to establish the disease status of contaminated animals in a relatively large number of individuals from the same species (Hÿvarinen and Sipilä, 1984; Siebert *et al.*, 1999; Jepson *et al.*, 2001). This complex approach became the warhorse of one group of scientists strongly backed up by an essential technical and administrative support: the **MARIN** Group (**M**arine **A**nimal **R**esearch and **I**ntervention **N**etwork). This multidisciplinary team, including veterinary pathologists, marine bird ecologists, ecotoxicologists and oceanographers, aims to study the potential causes of death and health status of the populations of seabirds and marine mammals found stranded along the Belgian and neighbouring coasts.

During the present work, we have considered the sources of variation in the trace metal concentrations of different marine mammal species from the North Sea and adjacent areas, as well as the Black Sea: geographic origin, species, age, sex or body condition. A special attention has been given to the influence of diet using the stable isotope approach ($\delta^{13}C$ and $\delta^{15}N$).

The potential role of trace metal concentrations (Zn, Ni, Cr, Cd, Fe, Cu, Se, Hg) as indirectly enhancing marine mammal mortality within the North Sea and adjacent areas was also examined. Due to sampling size, the case of the harbour porpoise (*Phocoena phocoena*) has been especially discussed. When available, trace metal levels were examined in relation with the general body condition observed at necropsy. Possible detoxification processes were investigated through the analyses of metallothioneins in livers and kidneys of selected harbour porpoises (*Phocoena phocoena*) and white-sided dolphin (*Lagenorhynchus acutus*).

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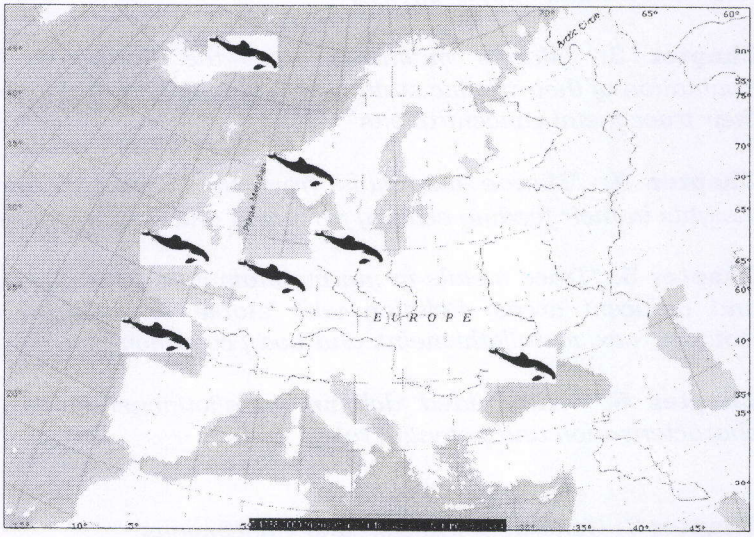
OBJECTIVES

The aim of the present work is to determine the relationships between trace metal levels and different factors such as species, geographic origin, diet, age and general body condition of the animals through:

- A systematic determination (and comparison) of trace metal levels (Zn, Ni, Cr, Fe, Cd, Cu, Se and Hg) in the livers, kidneys and muscles of different marine mammal species, mainly the harbour porpoise, *Phocoena phocoena*, but also the common dolphin, *Delphinus delphis*, the striped dolphin, *Stenella coeruleoalba*, the white-beaked dolphin, *Lagenorhynchus albirostris*, the white-sided dolphin, *Lagenorhynchus acutus*, the harbour seal *Phoca vitulina*, and the grey seal *Halichoerus grypus*. These animals have been collected within different parts of the Northeast Atlantic Ocean, the Irish Sea, the Southern North Sea and the English Channel, The German Baltic Sea, Danish, Norwegian and Icelandic coasts and the Black Sea.



Sampling area of this study

- A systematic analysis of isotopic composition -$\delta^{13}C$ and $\delta^{15}N$- in the livers and muscles of the above mentioned species in order to estimate and compare their respective trophic position.

- The study of the trace metal (Zn, Cd, Cu and Hg) speciation on metallothioneins in the livers and kidneys of selected animals (harbour porpoises and white-sided dolphins) from the North Sea.

- The investigation of the potential relationship existing between the trace metal levels in the tissues and the general body condition of the harbour porpoises.

The general introduction and results are presented in different chapters:

Chapter 1: The general introduction summarises some information concerning the North Sea, heavy metals, metallothioneins and stable isotopes in relation to marine mammals.

Chapter 2: "*Tuna and dolphin associations in the Northeast Atlantic: Evidence of different ecological niches from stable isotope and heavy metal measurements*"

Chapter 3: "*Marine mammals from the Northeast Atlantic : Evaluation of their trophic status by $\delta^{13}C$ and $\delta^{15}N$ and influence on their trace metal concentrations*"

Chapter 4: "*Marine mammals from the Southern North Sea: insights in their feeding ecology from $\delta^{15}N$ and $\delta^{13}C$ measurements*"

Chapter 5: "*Trace metals in marine mammals from the North Sea and adjacent areas: Relation with stable carbon and nitrogen isotopes, sex, metallothioneins and body condition*"

Chapter 6: "*White-sided dolphin metallothioneins: Purification, characterisation and potential role*"

Chapter 7: *General Discussion and Conclusions*

CHAPTER 1: GENERAL INTRODUCTION

1.1. THE NORTH SEA

1.1.1. OCEANOGRAPHIC FEATURES

The Northwest continental shelf area constitutes major fish-producing ecosystems in the world (figure 1.1). The North Sea is a semi-enclosed, epi-contiental large ecosystem and, as defined by the North Sea Task Force, includes the area south of 62°N (North Sea Task Force, 1993a;b), the Scandinavian straits (The Skagerrak and the Kattegat) and the English Channel (Ducrotoy et al., 2000).

The North Sea (or greater North Sea as defined by the OSPAR convention) has a mean depth of 90 m, opens broadly to the Atlantic and receives low-salinity water from the Baltic through the Kattegat and the Skagerrak, as well as from rivers. The North Sea basin is shallow, deepening to the North and is now heavily sedimented. Its western and northern coasts contain a variety of rocky and mountainous shores, sandy beaches, estuaries, and fjords, while the British east coast is bordered by cliffs, at places protected by pebbles, with a diversity of geological landscapes. On the eastern side, sand beaches and dunes prevail. The Wadden Sea offers large interdidal areas contained behind a girdle of barrier islands. The Wadden Sea area covers just 1% of the total, about half of which are tidal flats. These are important production and nursery areas for fish, birds or marine mammals (Sonntag et al., 1999; De Jong et al., 1999).

The topography of the North Sea produces a circulation influenced by the Coriolis forces. Waters entering from the north move to the western coast while those entering from the Channel move to the Dutch/Belgian coast (Turrel et al., 1992) creating a cyclonic (anti-clockwise) circulation. Currents are guided by topography and by bathymetry in the stratified Northern North Sea. The southern part of the North Sea is permanently mixed, while the middle and northern parts become stratified in summer.

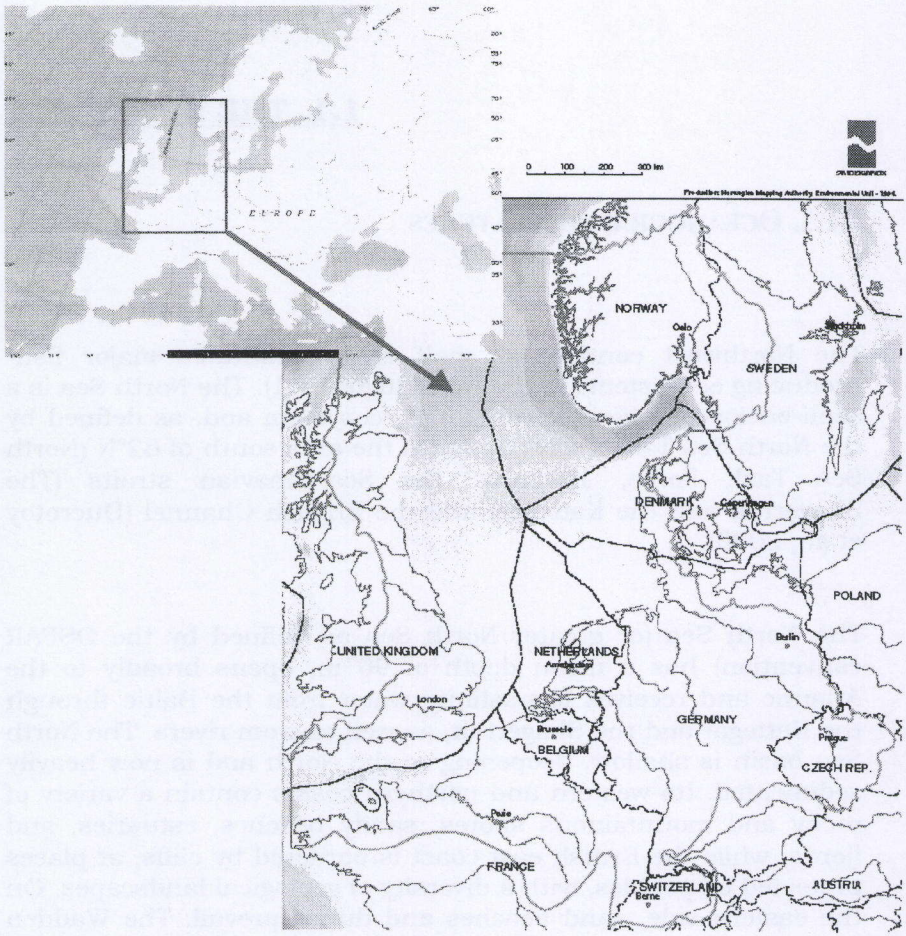


Figure 1.1. The North Sea area as defined in the context of the North Sea Conference (North Sea Secretariat, 1997b)

Depressed oxygen levels are found below 70 m where there is no wind influence. Flushing time for the entire North Sea has been estimated as one year to 500 days (Otto et al., 1990 quoted by Ducrotoy et al., 2000).

With an annual production in the range of 3 million tons, the North Sea contributes 3-4% of the world's fish production of approximately 100 million tons per year and is considered as the richest area in the Northeast Atlantic. Indeed, the production of the North Sea is associated with a relatively shallow depth and

the existence of mixing mechanisms transporting nutrients from the nutrient-rich bottom layer to the nutrient-poor upper layers of the water column.

Approximately 230 species of fish are known to inhabit the North Sea ranging in size from 5 cm gobies (*Pomatoschistus sp.*) to the 10 m basking shark (Cethorhinidae). Thirteen of these fish species are the main targets of major commercial fisheries: cod, haddock, whiting, saithe, plaice, sole, mackerel, herring, Norway pout, sprat, sandeel, Norway lobster, and deep-water prawn (*Nephrops sp., Pandalus sp.)* or brown shrimp (*Crangon crangon*). Norway pout, blue whiting, sprat and sandeel are predominantly the targets of industrial fisheries where the catch is converted into fish meal and oil while the other species are used for direct human consumption (Quéro and Vayne; 1997; Muus et al., 1998; OSPAR, 2000a; ICES, 2001b).
There are also landings from a variety of demersal species such as turbot, anglerfish, gurnards, lemon sole, rays, and sharks (ICES, 2001b).

Most common species are typical of a semi-enclosed shelf-sea, although deep-water species are found along the Northern shelf edge and in the deep-waters of the Norwegian Trench and Skagerrak. Different assemblages are found in relation to depth and substrate types.

In the Northern North Sea, on the slope edge, fishes are dominated by saithe *Pollachius virens* and haddock *Melanogrammus aeglefinus,* Norway pout *Trisopteris esmarkii,* whiting *Merlangius merlangus,* blue whiting *Micromesistius poutassou,* and cod *Gadus morhua* (Quéro and Vayne; 1997; Muus et al., 1998).

In the central North Sea, at depths of 50-200 m, the association is comparable but haddock dominates, together with whiting and cod. In shallower parts of the North Sea and the Channel, common dab *Limanda limanda* and whiting, together account for almost half of the biomass. Horse mackerel, *Trachurus trachurus* and sandeels *Ammodytes marinus* and *Hyperoplus lanceolatus,* also make a significant contribution to eastern and southern populations. Herring and mackerel *Scomber scombrus* show an interesting migratory spawning, between the North Sea and adjacent seas such as the Baltic Sea or Northeast Atlantic, where they use the continental slope (ICES, 2001b).

It must also be quoted that the North Sea is of global importance for seabirds feeding and breeding as 28 species with a total of 4.25 million seabirds breed and a further six species feed here (Dunnet et al., 1990; OSPAR, 2000a).

1.1.2. ANTHROPOGENIC IMPACT

Background

The North Sea is often considered as one of the most polluted seas in the world (Laane, 1992; North Sea Task Force, 1993a, 1993b, OSPAR, 2000b). Indeed, approximately 185 million people live in the highly industrialised countries bordering the North Sea. An additional 85 000 000 inhabitants occupy the Baltic catchment which flows into the North Sea (Ducrotoy et al., 2000). Densities of populations differ greatly, with over 1000 inhabitants per km^2 off the coasts of Belgium and the Netherlands to less than 50 inhabitants per km^2 along the coasts of Norway and Scotland. Tourism induces large scale seasonal migrations to the coast, amounting to millions of people on a single day.

Several large rivers (Thames, Humber, Elbe, Weser, Meuse, Rhine, Scheldt, Seine) loaded with contaminants (both dissolved and on suspended particulate matter) continuously discharge into the North Sea where they disperse according to the currents or accumulate locally in sedimentation areas such as the intertidal zones and estuaries (Pedersen, 1996; Radach and Keyer, 1997).

In addition to heavy anthropogenic inputs of pollutants through its main rivers, busy shipping routes and off-shore exploitations (gas and oil), the North Sea also provided waste incineration areas until early 1991, with subsequent high organochlorine residue levels in different studied benthic and pelagic organisms (Dethlesfen et al., 1996).

Figure 1.2. The North Sea contains some of the busiest shipping route in the world (picture from UGMM website **http://www.mumm.ac.be/**).

By far the largest industry in the North Sea, off-shore exploitations land more than 92. 10^9 m^3 of gas and 183. 10^6 tons of oil per year, which are transported through a 10 000 km long network of pipe-lines. Other activities, such as marine aggregate extraction, contribute to up to 15 % of some bordering country demands for sand and gravel. Serious physical impacts are related to substratum removal and alteration of the bottom topography, such as the formation of temporary plumes in the water column, and re-deposition of material (de Groot, 1996). Re-suspension of contaminated sediments, like those occurring during dredging activities, especially in harbours and their vicinity, largely influences pollutant distribution in the water column, making them readily bioavailable to invertebrate organisms (Fichet et al., 1998). The intensive use of the North Sea causes a number of problems in relation to a healthy ecosystem and sustainable use: a series of old problems continues to affect the ecosystem, sometimes showing some signs of improvement whereas new problems have also occurred (i.e. detection of new synthetic compounds) (OSPAR, 2000b).

Fishing activities have 3 main effects on North Sea ecosystems.

The first is due to direct fishing pressure. Capture of fish and shellfish leads to mortality of both target and non-target species. The long term effects of high fishing mortality have

resulted in a decrease in abundance in older age groups of target species. At various time during the last ten years, the North Sea stocks of cod, haddock, whiting, saithe, plaice, and herring have dropped to or below any previously recorded level (5NSC, 1997). The spawning stock of mackerel has not yet recovered since its collapse in the mid-1960s. In the case of cod, haddock, and herring, recovery has been in evidence in recent years. However all the major stocks of roundfish and flatfish and also the herring stock are considered by ICES to be close to or outside safe biological limits.

A second effect of fishery on the North Sea ecosystems is due to the practise of discarding which can significantly impact the ecology of scavengers and predators.

Finally, there are direct and indirect effects on benthic communities through direct physical disturbance of sediment by trawling as well as increased organic input derived from discard fish and invertebrates (OSPAR, 2000b).

Contaminants within the North Sea

Considerable efforts to understand the biogeochemistry of pollutants in the marine environment have been made over the last decades. The fate of contaminants in the North Sea is closely linked to their distribution between the dissolved and the particulate phase. In general, dissolved substances will follow the movements of the respective water masses. Due to their strong association with particulate matter, the fate of many contaminants in the Greater North Sea is, however, related to the transport of (re) suspended solids. In areas of deposition, sediments are loaded with contaminated particles, and it is estimated that 70% of the substances associated with particulate matter remain trapped in sedimentation area such as estuaries, the Wadden Sea, the deepest part of the Kattegat-Skagerrak and the Norwegian Trench (Laane et al., 1998).

Metals have entered the North Sea ecosystem since historical times, naturally or due to human activities. Pollution from metal mining in the Pennine ore fields has induced contaminant fluxes to the North Sea for centuries. Industrial pollution and run-off from urban areas are other important contributors of metal inputs as well as atmospheric sources.

Heavy metals reach the North Sea via both airborne and waterborne inputs. Inputs are also generated by some sea-based activities, such as exploitation of offshore resources and dumping of dredged materials. Typical atmospheric deposition levels at around 500 km from the coast are an order of magnitude lower than deposition into coastal waters close to industrialised areas. The riverine fluxes of metals consist of particulate and dissolved species but the particulate phase is predominant for most elements. The spatial distribution of metals in the coastal zone, where highest concentrations are found near fresh water outlets and lowest in the open sea, suggests that rivers are major sources of trace metals. However, the dumping of dredged material can disturb this pattern locally (OSPAR, 2000b).

Many studies either used sediments or fishes as chronological indicators (Jorgensen and Pedersen, 1994; Pedersen, 1996; Callaway et al., 1998; Laane et al., 1998). A decline in the concentration of various metals has generally been observed in the surface sediment in dynamic sediment zones. This is due to

mixing with less contaminated suspended matter or the winnowing of contaminated particles.

The recent history of heavy metal accumulation within the North Sea can be observed in (net) deposition areas such as the Skagerrak and Norwegian Trench. In the deepest part of the Skagerrak, Hg concentration in sediments have increased significantly since about 1950, while increases in Pb and Cu concentrations began somewhat earlier, around 1900. In the North Sea, atmospheric inputs of Pb decreased by 50 – 65% between 1987 and 1995 such that the dominant source is now considered to be rivers. Decreasing atmospheric Pb inputs are reported from several regions of the Northeast Atlantic and adjacent areas and these have been attributed to the reduction in use of alkyl Pb derivatives in petroleum (OSPAR, 2000a,b).

Apart from the changes in the contaminant load to these areas over recent decades, a number of other factors have influenced the observed pattern, including variations in sediment transport and erosion rates, eutrophication and environmental changes in the deeper part of the Skagerrak (OSPAR, 2000a,b). However, estuaries and their surroundings, receiving large input of metals from industrial sources, are still identified as areas where metal concentrations in sediments are clearly elevated. It has for instance, been estimated that between the Dutch-Belgian border and the open North Sea roughly half of Cd and almost 90 % of the PCB loads ends up in the sediment of the Western Scheldt.

In general, risks to ecology of the North Sea from metals appear to stem from Cu accumulation (mainly due to effects on the production at lower trophic levels such as bacteria and phytoplankton), Cd and Hg (for top-predators), and Pb (on predators of shellfish). These effects are expected to occur most frequently in estuaries and in the coastal zones. They are due in large part to the tendency of these metals to bioaccumulate in organisms through trophic interactions (OSPAR, 2000a,b).

- **Cadmium (Cd)**

  Atmospheric deposition of Cd is responsible for about one third of the total Cd input into the greater North Sea while waterborne inputs constitute the remainder. Total Cd input

reaching the Greater North Sea is estimated between 79 and 118 t/y depending on the year or the model used (OSPAR, 2000a,b). Large decreases of dissolved Cd have been observed in the North Sea these last 20 years, especially in the Southern Bight and in the Dutch coastal zone, the Thames estuary and the Dogger Bank area (Scholten et al., 1988). In the sediment, the Cd content seems also to have decreased in areas with previously high concentrations such as the Dutch coastal zone, the mouth of the Rhine and Meuse rivers, the Schelde estuary and the Wadden Sea. No significant decrease was found 20-70 km offshore (Laane et al., 1998). Last 20 year-trend in the biota was examined in blue mussels and significant downward trends were found in blue mussels and flounder livers from the Netherlands and Norway and in cod livers from Sweden (OSPAR, 2000a,b).

However, Cd concentrations in biota seldom exceed the Background Reference Concentration (BRC which is assumed to reflect concentration before human contamination or for pristine areas) except in punctual locations such as estuaries (Tay, Forth, Seine) or Norwegian fjords (Sørfjord, due to the presence of smelting industries). In the Sørfjord (Norway), there is still advice against consumption of blue mussels due to high Cd (and Pb) levels (OSPAR, 2000b).

- **Mercury (Hg)**

Riverine and direct inputs of Hg to the Greater North Sea ranged from approximately 7.6 to 9 t/y in 1996. Calculated atmospheric inputs was estimated to 4t/y in 1995. Combined riverine inputs from the Rhine, Meuse and Elbe rivers dominate (OSPAR, 1998). Since the mid-1980s, technical improvements in production technology have lead to a significant decrease in Hg emissions. This is also due to important Hg emitters in the former German Democratic Republic being closed down. Hg in water ranges from 1 ng/l at the mouth of estuaries to 0.1 ng/l in open seawater. Particulate Hg accounts for over 90% of the total Hg in coastal waters and decreases to less than 10% in the less turbid open sea (OSPAR, 2000b). Dissolved and toxic monomethyl-Hg concentrations may be more than 60 times higher in estuaries compared to open sea (600 pg/l *vs* 10-60 pg/l respectively).

In the sediment the higher concentrations are found in the estuaries of the Scheldt, Forth, Elbe, and Thames as well as near-shore stations and dredged spoil disposal sites. In the Belgian coastal area, an annual 5% decrease was noted between 1979-95 in some sewage disposal sites.
In the biota, significant downward trends have been observed in flounder, plaice and blue mussels from the Southern North Sea.
However, blue mussels from industrial areas of Norway display high Hg concentrations with respect to BRC. Enrichment factor (mean concentration at site divided by the BRC) up to 3 can be observed near the German, Belgian and Dutch coasts in some cod or dab species.

- **Copper (Cu)**

Cu waterborne inputs in the greater North Sea region were estimated around 1100t/y in 1996 while model calculations give an estimate of 56t/y of Cu reaching the Greater North Sea via atmospheric deposits (OSPAR, 2000b). No clear decrease either for atmospheric or waterborne Cu inputs have been observed these last 10 years party due to the relative importance of diffuse sources. However a general decrease has been observed in the sediment of the Dutch coastal zone, the mouths of the Rhine and Meuse, in offshore areas with the highest decrease noticed for the Belgian coast with concentrations dropping by 65% between 1990 and 1995. In the biota, decreasing patterns have been shown for mussel and fish tissues from Denmark, Germany, Netherlands as well as in the Elbe estuary (which is related to the decline of chemical industries in the former GDR). Background Reference Concentrations are exceeded in some specific locations such as the Scheldt, the Tay and the Forth estuaries (OSPAR, 2000b).

A general loss of biodiversity in seaweeds and seagrasses as well as in benthic communities was observed, with a progressive but consistent shift towards opportunistic species (Philippart, 1998 ; Ducrotoy, 1999; Frid and Hall, 1999; Lindeboom and de Groot, 1999; Wolff, 2000a; 2000b) .

1.1.3. PROTECTION AND MANAGEMENT

Protection and management of the seas and oceans have greatly developed during the last 30 years. First concerned by the management of the fisheries resources and the prevention of oil pollution, international agreements soon progressed towards a global protection of the marine environment.

- In the North Sea, a first international agreement regarding the oil pollution was signed in the late 1960s (*Agreement for Co-operation in Dealing with Pollution of the North Sea by Oil*, Bonn, June 1969).
- Soon followed the *Oslo Convention* (*Prevention of Marine Pollution by Dumping from Ships and Aircraft*, 1972) implementing regulations for dumping waste at sea.
- The next step was to prevent marine pollution from land-based sources, an agreement signed at Paris in 1974 (*Paris Convention for the Prevention of Marine Pollution by Landbased Sources*).
- Several international conferences then took place (Bremen, 1984; London, 1987; The Hague, 1990; Copenhagen, 1995; Lisbon, 1998) to co-ordinate, evaluate and re-adjust this international joint effort towards the protection, preservation and sustainable management of the North Sea environment (Tromp and Wieriks, 1994).
- All coastal states around the North Sea have ratified the *Ramsar Convention on Wetlands of International Importance*. Many of these wetlands (*i.e.*: the Flemish Banks situated within the Belgian area of interest, 51-52° N°), together with other sites, qualified as *Special Protection Areas* under the EC Directive on the Conservation of Wild Birds, 1979 (Carter et al., 1993; Jacques, 1995).
- In addition to the *Ramsar Convention*, Belgium has also ratified the *Agreement on the Conservation of Small Cetaceans of Baltic and North Sea* (ASCOBANS).
- Furthermore, in accordance with the *European Commission Habitats Directive*, Belgium has also proposed a large part of the western part of the coast to be included in the Natura 2000 network as a 'Special Area for Conservation'.

1.2. MARINE MAMMALS IN THE NORTH SEA

The fertile waters of the North Sea represent one major life site for at least four different marine mammal species using this habitat for breeding and feeding: the harbour porpoise (*Phoceona phocoena)*, the harbour seal (*Phoca vitulina*), the grey seal (*Halichoerus grypus)* and the bottlenose dolphin *(Tursiops truncatus)*. The North Sea bottlenose dolphin population is located in the Moray Firth in Scotland and seems to be the only one left within the North Sea since 1937 (Wolff, 2000a; 2000b).

Table 1.1. Estimated number of seals, harbour porpoises, dolphins and minke whales in the North Sea (Hammond et al., 1995; OSPAR, 2000b; Wolff, 2000a; 2000b;)

Species	Location	Number
Harbour seal *Phoca vitulina*	West coast and North Sea coast, Oslofjord (Norway)	3400
	Orkney, Shetland	14100
	East Scotland and East England (UK)	1700
	Wadden Sea coast	7040
	Danish Limfjord	700
	Kattegat/Skagerrak	6300
Grey seal *Halichoerus grypus*	Norway coastline	2100
	UK North Sea coast	58300
	Wadden Sea coast	250
	Kattegat	<25
Harbour porpoise *Phocoena phocoena*	North Sea	268 300
Minke whale *Balaena acutorostrata*	North Sea	7200-20 000
White-beaked/ White-sided dolphin *Lagenorhynchus albirostris/ L. acutus*	North Sea	10 900
Bottlenose dolphin *Tursiops truncatus*	Moray Firth (Scotland)	Few

The harbour porpoise is by far the most common species in the Northeast Atlantic and the North Sea (Hammond et al., 1995). The harbour porpoise is a small (2 m and 50 kg) coastal cetacean

found over the continental shelves of the temperate northern hemisphere (Read, 1999). The species is relatively short lived (few animals live longer than 15 years), attains sexual maturity at age 3 or 4, and gives birth to a single offspring every year, or every second year, in early summer (Read, 1990a; 1990b).

Harbour porpoise represents a major component of the North Sea food web with an estimated annual fish consumption of approximately 60 000 tons (by comparison, humans' fish consumption is around 2.5 x 10^6 tons) (Bjôrge and Øien, 1990 quoted by Hislop et al., 1992). This species is known to be incidentally taken by fishing vessels both in the North Sea and Baltic Sea (ICES, 2001a; Berggren et al., 2002). It used to be a very common species in Dutch coastal waters, but it has disappeared from these waters since the 1960s (Addink and Smeenk, 1999; Wolff, 2000a; 2000b). The species seems now absent from the Dutch Delta area and from the Dutch and German Wadden Sea west of the River Elbe (Vervey and Wolff, 1981a,b). The first census of marine mammals in the North Sea and the Channel (SCANS) showed conspicuous absence of harbour porpoises in the channel and the southern part of the North Sea (Hammond et al., 1995). Nevertheless, the species continues to strand on Dutch beaches, although presumably in much lower numbers than before the second World War (Addink and Smeenk, 1999). So, although the harbour porpoise does not occur in inshore waters any longer, there apparently still exists a small population off the Dutch coast (Wolff, 2000a; 2000b). Moreover, the increase of porpoise strandings these last four years along the Northern French, Belgian and Dutch coasts indicates the presence of this species in the area (Jauniaux et al., 2002).

The harbour porpoise status in the southern part of the North and Wadden Seas remains unclear: this species seems to have declined severely in the Wadden Sea and its inlets during World War II and has remained rare since then (reviewed by Addink and Smeenk, 1999). In the beginning of this century, harbour porpoises were found throughout the entire North Sea around the UK and Denmark, off France and Belgium, the Netherlands, Germany, in the southern, central and northern North Sea, the Kattegat, the Baltic, in Swedish and Norwegian waters, up to the Barents Sea and the White Sea (Reijnders, 1992). Recent studies have provided evidence that the harbour porpoise of the Southern North Sea form a separate sub-population (quoted by Addink and Smeenk, 1999) and it is concluded that the southern North Sea

porpoises should be considered separately from other populations.

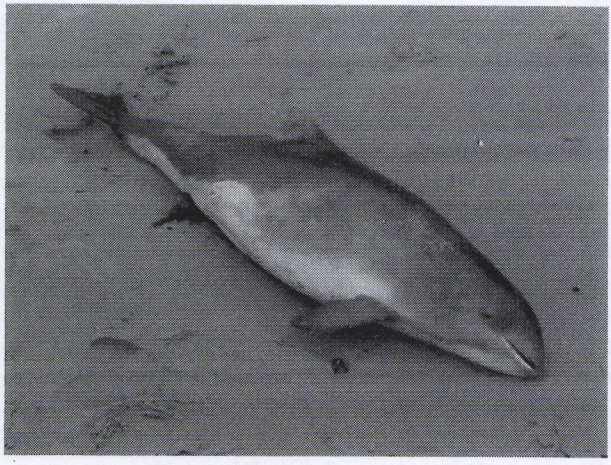


Figure 1.2. Harbour porpoise, *Phocoena phocoena* is the main species stranded along the Southern North Sea coast (picture kindly provided by J. Haelters, BMM)

The white-beaked dolphin (*Lagenorhynchus albirostris*), the white-sided dolphin (*Lagenorhynchus acutus*) and the minke whale (*Balaenoptera acutorostrata)* occur regularly over large parts of the North Sea in large number mainly to feed (ICES, 2001a).

White-beaked dolphins are generally concentrated in a band across the North Sea between 55° and 60° N, mostly to the west along the eastern British coast (Hammond et al., 1995). However, extended movement might occur as well, as individuals have been observed in the Southern North Sea and collected stranded on the Belgian and Dutch coasts (Haase, 1987 ; J. Seys, pers. comm.).

Harbour seal is one of the most widely distributed seal in the world and the North Sea contains around 10 % of the world population (North Sea Task Force, 1993a,b). Counts of common seal numbers (1994-1996) estimate the current North Sea population at 36 000 seals. The harbour seal and the grey seal breed along the coast of the North Sea. An epidemic of phocine distemper virus in 1988 significantly reduced their numbers but

by the late 1990s they recovered. Since 1989, numbers have increased to more than 14 000 (Reijnders and Reineking, 1999).

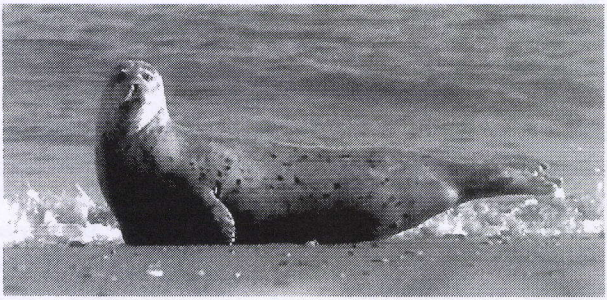


Figure 1.3. The harbour seal, *Phoca vitulina* (picture from Wandrey, 1999)

Some species occur in the North Sea on an occasional or temporary basis. Hooded seal (*Cystophora cristata)*, killer whale (*Orcinus orca*), long-finned pilot whale (*Globicephala melas*), Sowerby's beaked whale (*Mesoplodon bidens*), northern bottlenose whale (*Hyperodon ampullatus*) and Risso's dolphin (*Grampus griseus*) regularly enter the northwest entrance to the North Sea (ICES, 2001a).

More occasionally, the larger whales including the sperm whale (*Physeter macrocephalus*), sei whale (*Balaenoptera borealis*), fin whale (*Balaenoptera physalus*) and the blue whale (*Balaenoptera musculus*) also approach the border of the North Sea. The common dolphin (*Delphinus delphis*) and striped dolphin (*Stenella coeruleoalba*) frequently enter the northwest North Sea entrances and the southern English channel water (Camphuysen and Winter, 1995 ; Hammond et al., 1995; ICES, 2001a).

The North Sea is a complex ecosystem made of sand banks, mudflats, sandy islands and estuaries making it unfavourable to such oceanic species (Hammond et al., 1995). Indeed, winter-stranded sperm whales show an absence of food in the gut, weight loss and blubber thickness reductions compatible with an extended presence in the North Sea where food is inadequate (Jauniaux et al., 1998).

Marine mammals have been exploited for centuries for subsistence purposes by human populations on all continents except Antarctica. Historical evidence indicates that cetaceans were abundant in the North Sea and in the English Channel

during the Middle Ages and earlier. Right whales and perhaps gray whales probably were hunted regularly in this area from at least the ninth century onward; harbour porpoises and other small cetaceans were hunted in this area before the sixth century. The impact on the populations was apparently localized and small in scale in comparison to later industrialized commercial hunting (Smet, 1981).

Although hunting has stopped and legal protection increased (IWC, Marine Mammals Protection Act –USA-, Ascobans,...) the populations are currently far from being unaffected by human activities, such as disturbance, change in physical habitats and/or destruction of habitats, oil spill damages, net entanglements, interactions with fisheries and environmental pollution. Incidental entanglement and mortality in fishing gear is a global problem affecting many species of small cetaceans. The effects of by-catch are probably the most important adverse impact to small cetaceans, especially to harbour porpoises (De Jong et al., 1999, OSPAR, 2000b; Berggren et al., 2002). Taking into account abundance estimates, estimated by-catch, and harbour porpoise biology, it is likely that the current by-catch alone poses a significant risk to the North and Baltic Seas populations (OSPAR, 2000b; Berggren et al., 2002). In addition to entanglement, an unknown number of marine mammals are also killed by discarded or lost fishing gear worldwide (Berta and Sumich, 1999, Ospar, 2000b).

There appears to be an overall increase in cetacean strandings in the North Sea which by hypothesis has been related to an overall increase in population size for some species, particularly sperm whales (*Physeter macrocephalus*), due to their protected status. Groups of male sperm whales occasionally visit the North Sea, particularly in the period between November and March during their southward migration, and strandings have been most frequent during these months (Smeenk, 1997).

Occasionally, group strandings typically occur in the southern region where the coastal topography is characterised by extensive sandbanks, mudflats and estuaries. Fossil data indicate that, though relatively rare, this phenomenon has been occurring for thousands of years (De Smet, 1997).

1.3. HEAVY METALS IN MARINE MAMMALS

(After: Das, K., Debacker, V., Pillet, S., Bouquegneau, J.M. 2002. In: Toxicology of Marine Mammals (ed. Vos, G., Bossart, G., Fournier, M., O'Shea, T.) Taylor and Francis Publishers Washington D.C. 600 pp. *in press*

1.3.1. INTRODUCTION

During the past few decades, increasing concern about environmental pollution has led to many investigations on heavy metals and their distribution in the sea, air or biological materials. The distribution of xenobiotics in the marine environment is not homogeneous and a considerable variation of concentrations may occur regionally and temporally. The use of bioindicators offers a useful alternative for pollution monitoring studies. Marine mammals appear to be potentially valuable indicators of the level of heavy metals accumulated in the marine environment: due to their top position in the trophic

network, their long life span and their long biological half-time for the elimination of pollutants, these animals accumulate high levels of chemicals, such as organochlorines (Tanabe et al., 1994; Kamrin and Ringer, 1994), or heavy metals (André et al., 1991a; Bouquegneau and Joiris, 1988; 1992; Dietz et al., 1998).

The increased interest in studying contaminants in marine mammals is due to large-scale die-offs (Sarokin and Schulkin, 1992; Forcada et al., 1994) or impaired reproduction (De Guise et al., 1995), which could lead to population declines of some pinniped and small cetacean species in Europe and North America, and the finding of relatively large contaminant burdens in these animals. In many cases, morbillivirus infections were the primary cause of the disease outbreaks (Heide-Jørgensen et al., 1992; Thompson and Hall, 1993; de Swart et al., 1995a). These mass mortalities among seals and dolphins inhabiting contaminated marine areas have led to speculation about the

possible involvement of immunosuppression associated with environmental pollution.

Controlled experiments are unavailable to establish any definite causal relation between these pollutant concentrations and any physiological problem. Moreover, the data are always difficult to interpret because of the presence of other chemical contaminants and other stressors. The physiological status of the organisms (e.g. pregnancy, moulting, fasting...) also modulates the toxicity of heavy metals. In addition, the available measurements have almost all been performed on animals found dead, which leaves doubts about the general applicability of collected data at which an effect at individual or population level might be expected.

Several investigations have been carried out in an attempt to evaluate contaminant effects at ambient environmental levels (Reijnders, 1986; Aguilar and Borrel, 1994; De Guise et al., 1995; de Swart et al., 1995b; 1996). For example, it has been demonstrated that seals fed polluted fish from the Dutch Wadden Sea showed reduced pup production when compared to those fed much less polluted fish from the Northeast Atlantic (Reijnders et al., 1986). This study was the first sign of a causal relationship between naturally occurring levels of pollutants and a physiological response in marine mammals.
A more recent study over a two year period by de Swart et al. (1994; 1995b; 1996) has demonstrated an impairment of several immune parameters in harbour seals (*Phoca vitulina*) fed on herring from the polluted Baltic Sea when compared to those fed with fish from the Atlantic Ocean.
Among impaired parameters, natural killer cell activity plays an important role in the first line of defence against viral infections (de Swart et al., 1996). Moreover, those seals consuming contaminated herring accumulated higher body burdens of potentially immunotoxic organochlorines than seals fed relatively uncontaminated herring. In the latter study, heavy metal levels have unfortunately not been determined either in fish or in seals. Possible immunosuppressive actions of other groups of environmental contaminants, such as heavy metals, cannot be ruled out. Indeed, many laboratories and epidemiological studies have demonstrated the immunotoxic effects of heavy metals in a variety of species (Zelikoff and Thomas, 1998). Direct cause and effect links between a single kind of contaminant and possible population declines has not been established so far, so that many researchers have proposed the possibility of a synergistic role of

different substances in increasing the susceptibility of affected animals to diseases or biotoxins.

In this chapter we will focus on heavy metals and their possible effects on marine mammals. Heavy metals are usually divided into essential (Zn, Cu, Cr, Se, Ni, Al) and non-essential metals (Hg, Cd, Pb), the latter being potentially toxic even at low concentrations. Nickel and Cu hazards to wildlife have been extensively reviewed by Eisler (1997; 1998). When considering marine mammals, there are limited data about heavy metals, except for Hg. Chromium, nickel and lead concentrations are generally low, rarely exceeding a few $\mu g.g^{-1}$ dry weight (dw) in marine mammal tissues. No meaningful assessment of their toxicity in marine mammals can be made as yet (Law, 1996). However, investigations carried out on a ringed seal population *(Pusa hispida saimensis)* from Finland showed a clear connection between stillbirth of the pups and nickel concentrations in the air (Hÿvarinen and Sipilä, 1984). These authors have underlined the considerable nickel input in the environment from industrial activity in that particular area. On the other hand, Zn, Cu, Cd and Hg concentrations often exceed several tens $\mu g.g^{-1}$ dw and so will be discussed in particular as well as their levels in the different marine mammal groups, detoxification mechanisms, potential hazards and ecological implications.

1.3.2. FACTORS AFFECTING HEAVY METAL CONCENTRATIONS

Some reviews about heavy metal contamination of marine mammals have been published (Wagemann and Muir, 1984; Thompson, 1990; Kemper et al., 1994; Law, 1994; 1996). Tables A1 to A5 (in appendix) present some selected concentration values and respective references in Odontocetes, Mysticetes, Pinnipeds, Sirenians and polar bears. It appears that metal concentrations vary greatly within marine mammals, especially for non-essential metals such as Cd and Hg (**table 1.1**).

Table 1.1. Examples of maximum and minimum metal concentrations for marine mammals. When available, the age of the animals is given (all the data are expressed in $\mu g.g^{-1}$ dry weight, assuming a mean water content of 75% of the tissue).

Metal	Minimum	Maximum
Hg	**0.2** muscle *Pusa hispida* Western Arctic Wagemann et al., 1996	**13 156** liver *Tursiops truncatus* Mediterranean Sea Leonzio et al., 1992
Cd	**0.007** muscle *Pusa hispida* (<1y.) Northwest Greenland Dietz et al., 1998	**2324** kidney *Pusa hispida (8y.)* Northwest Greenland Dietz et al., 1998
Zn	**2** blubber *Leptonychotes weddellii* (13 y.) Antarctic Yamamoto et al., 1987	**4183** liver *Dugong dugong* (>30 y.) Australia Denton et al., 1980
Cu	**0.4** blood *Leptonychotes weddellii* (13 y.) Antarctic Yamamoto et al., 1987	**600** liver *Tursiops truncatus* Argentina Marcovecchio et al., 1990

The large variation of these data (table 1.1 and A1 to A5) illustrates the numerous physiological and ecological factors that might affect heavy metal contamination: geographic location, diet, age, sex, the tissues considered and metabolic rates. Information concerning metabolic rates is largely unavailable for the different marine mammals species so will not be discussed here. However it must be kept in mind that ingestion and assimilation rates differ between all the marine mammal species in relation with their weight and their migration or physiological status (fasting).

1.3.2.1. Geographic location

Heavy metal contamination sources can be both anthropogenic and natural and distinguishing between the two can be very difficult. The natural background component of the input of heavy metals in marine ecosystems may be as important as the anthropogenic one, and in some areas it appears to be the major source. This is important because it emphasises that marine mammals have been exposed to heavy metals long before the development of human activities. It is *e.g.* the case for the Mediterranean Sea and the Arctic, which are known for their high natural metal levels: Hg in the Mediterranean Sea and Cd in the Arctic.

The Hg levels measured in dolphins from the Mediterranean Sea are higher than those encountered in dolphins from the Pacific coasts of Japan or the Northeast Atlantic (figure 1.5) (André et al., 1991a; Honda et al., 1983; Leonzio et al., 1992). According to André et al. (1991b), the origin of high Hg levels observed in Mediterranean dolphins is certainly natural because of the large natural sources present in the Mediterranean basin.

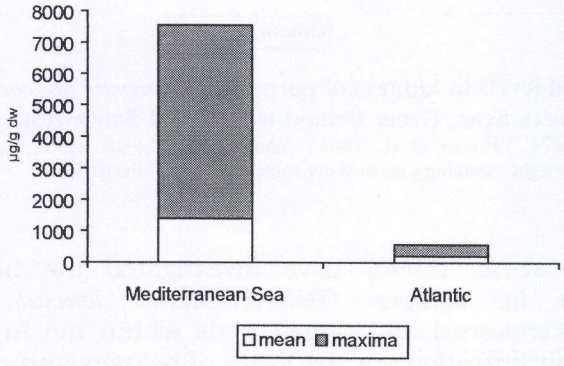


Figure 1.5. Mercury levels in livers from striped dolphins (*Stenella coeruleoalba*) from the Northeast Atlantic and the Mediterranean Sea (After André et al., 1991a;1991b). Concentrations are expressed in dry weight assuming a mean water content of 75% of the tissues.

The current state of knowledge of concentrations, spatial and temporal trends of contaminants including heavy metals have

been extensively described in the Arctic (Muir et al., 1992; Dietz, 1996; AMAP, 1998). It seems that Cd concentrations have always been high in the Greenland Arctic regions as indicated by the lack of obvious temporal trends in sediment cores, as well as historic hair samples from the 15th century from both seals and Inuits (Dietz et al., 1998). Johansen et al. (1980) first reported that Cd levels in tissues of ringed seals from Greenland were higher than previously reported for seals from the North Sea. Cd seems to accumulate to higher levels in seals living in unpolluted Arctic waters than those taking their preys in the North Sea area, which receives large inputs of pollutants. A similar observation can be made for porpoises (figure 1.6).

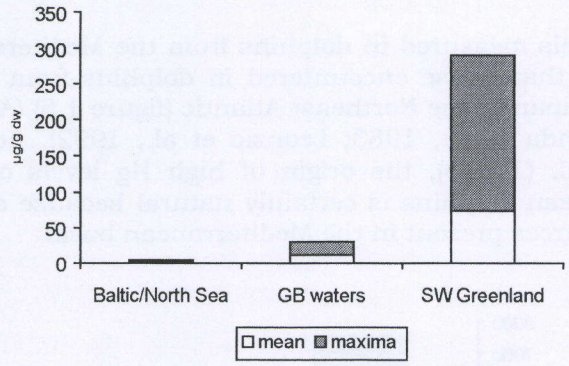


Figure 1.6. Cd levels in kidneys of porpoises (*Phocoena phocoena*) from the Baltic and North Seas, Great Britain waters and Southwestern Greenland (Harms et al., 1978; Falconer et al., 1983 ; Paludan-Müller et al., 1993). Concentrations are expressed in dry weight assuming a mean water content of 75% of the tissues.

Wagemann et al. (1996) have investigated the heavy metal distribution in belugas (*Delphinapterus leucas*), narwhals (*Monodon monoceros*) and ringed seals within the Arctic region. Mean Hg concentrations in the livers of belugas and ringed seals were significantly higher in the Western Arctic than in the Eastern Arctic. This comparison was not possible for narwhals, as they are not found in the Western Canadian Arctic. On the contrary, Cd as well as Zn and Cu concentrations in tissues (liver and kidney) of belugas and ringed seals were higher in the Eastern than in the Western Arctic. These differences in metal levels between marine mammals of the Eastern and Western

Arctic corresponded to the different geological settings and sediment of these two regions (Wagemann et al., 1995; 1996).

1.3.2.2. Routes of entry

There exist several different routes of entry of heavy metals in marine mammals: uptake from the atmosphere through the lungs, absorption through the skin, across the placenta before birth, via milk through lactating, ingestion of sea water and ingestion of food. Nevertheless, the major route of heavy metal contamination for marine mammals seems to be via feeding (André et al., 1990a,b; Augier, 1993b; Law, 1996). Considering that, the following discussion will mainly refer to the diet (including suckling) and its influence on metal uptake.

Obviously, mysticetes are less contaminated by heavy metals than odontocetes and pinnipeds as these are located at higher trophic levels in the marine food web. Cd seems to be transferred to the highest trophic levels of the marine food chain mainly by molluscs, particularly cephalopods, which concentrate Cd in their viscera (Honda and Tatsukawa, 1983; Bouquegneau and Joiris, 1988; Miles and Hills, 1994). Long-finned pilot whales (*Globicephala melas*) for example are known to concentrate Cd in relation with a preferential cephalopod diet. Elevated levels of Cd in Pacific walrus (*Odobenus rosmarus divergens*) and northern fur seals (*Callorhinus ursinus*) have been reported in a population from the Bering Sea, a remote area away from industrial activity (Miles and Hills, 1994). In an attempt to find out which prey may transfer Cd to walrus, the most common preys (mainly bivalves) found in the stomach contents were analysed for their metal concentrations. Amongst the bivalves analysed, *Mya sp.* showed the highest Cd content suggesting that this prey could be a Cd transmitter for walrus (Miles and Hills, 1994). Wagemann and Stewart (1994) have studied heavy metal concentrations of walrus (*Odobenus rosmarus rosmarus*) from the Eastern Canadian Arctic in relation with their food, mostly bivalves (*Mya sp.* and *Serripes sp.*). These authors showed that bivalves could also be a source of lead for walrus, judging the correspondence between the high levels of Pb in both bivalves and walrus (Wagemann and Stewart, 1994).

However, some high Cd levels have been reported in certain marine mammals that do not eat Cd-contaminated prey (Denton et al., 1980). Trace metals have been determined in dugong,

Dugong dugong, which mostly feeds on seagrasses and algae. High Cd and Zn concentrations have been detected in their liver and kidney. Denton et al. (1980) have reported renal Cd and hepatic Zn concentrations reaching respectively 309 $\mu g.g^{-1}$ and 4183 $\mu g.g^{1}$ dry weight while low levels of these metals are found in the seagrasses analysed. The authors suggested that the low levels of Cu in seagrasses may influence intestinal absorption of Zn and Cd in the dugong. Higher than normal Zn and Cd absorption through the intestinal tract occurs when dietary Cu intake is deficient, due to competition between the metals for available binding sites on carrier proteins within the intestine (Denton et al.,1980). Unlike dugongs of coastal Australia which display a Cu deficiency due to seagrass diet, Florida manatees (*Trichechus manatus*) are considered to be facing the opposite problem: Cu concentrations in the livers of Florida manatees were significantly elevated in areas of high herbicidal Cu usage (O'Shea et al., 1984). Manatees might be inefficient at maintaining Cu homeostasis in the face of dietary excess. The death of a captive dugong was attributed to exposure to Cu sulphate added to its tank as an algicide (Oke, 1967).

The position of top predators in the food web influences Hg levels in marine mammals as this highly toxic metal is biomagnified, when available as methylmercury, through the food web (Bouquegneau and Joiris, 1988). Diets, and especially those of marine mammals relying on fish, are responsible for Hg contamination (Svensson et al., 1992; Nakagawa et al., 1997). Much higher Hg concentrations have been reported in minke whales (*Balaenoptera acutorostra)* from Greenland (Hansen et al., 1990) compared to Antarctic minke whales (*Balaenoptera bonaerensis*), which are several times less contaminated (Honda et al., 1986; 1987). Hansen et al. (1990) attributed the lower Hg concentrations found in tissues of Antarctic minke whales to differences in trophic levels and subsequently in the Hg content of the food items: Northern minke whales feed mainly on fish while Southern minke whales feed on krill. Indeed, the food web in the Antarctic ecosystem is rather simple, as the major food component is the Antarctic krill. The low trophic position of the Antarctic minke whale is reflected in the very low bioconcentration factor of Hg in this animal.

1.3.2.3. Age

Cd and Hg accumulated strongly with age in most marine mammals tissues analysed (*e.g.*: Hamanaka et al., 1982; Honda and Tatsukawa, 1981; 1983; Honda et al., 1983; Augier et al., 1993b). This increase is enhanced in the liver in which the excretion rate might be extremely low due to the fossilisation of Hg under a detoxified form (see below). However, some exceptions have been reported in the literature. Honda et al. (1986; 1987) have studied the heavy metal distribution in Antarctic minke whales, and compared it to their food habit and age. The age of these whales varies between 1 and 45 years. They found maximum concentrations of Cd and Hg in the livers of 20 year old minke whales. Both concentrations increase with age until about 20 years and thereafter decrease. Because there is no evidence that half-lives of Cd and Hg change with age, Honda et al. (1986; 1987) suggested a higher food intake for the younger animals compared to older. They also suggested that these changes may be due to the significant decrease in stocks of blue whales (*Balaenoptera musculus)*, and fin whales, *(Balaenoptera physalus)*. Both species used to occupy ecological niches similar to those of the minke whales in the Antarctic marine ecosystems. The young minke whales would be less exposed to feeding competition from blue and fin whales, than the older ones. Accumulations of toxic metals such as Cd or Hg may also have been influenced by this ecosystem disruption (Honda et al., 1986; 1987).

High Cu and Zn concentrations have also been observed in very young animals and neonates (*e.g.*: Julshamn et al., 1987; Wagemann et al., 1988; Caurant et al., 1994). These essential elements are known to increase in tissues undergoing rapid development and differentiation (Baer and Thomas, 1991). It has been suggested that these higher levels encountered in young might reflect a specific requirement in newborns or a very low excretion rate of these metals by the fœtus (Wagemann et al., 1998).

1.3.2.4. Sex

Reproductive activities such as pregnancy, parturition and lactation can modify metal levels. Several studies have demonstrated that metal transfer from females to pups occurs through the placenta or lactation: Honda et al. (1987) reported a

hepatic Fe, Co, Pb and Ni transfer from mother to pup. As a consequence, these metal concentrations decrease in the mature female with progress of gestation.
Canella and Kitchener (1992) found significantly lower levels of Hg in pregnant and lactating sperm whales (*Physeter macrocephalus)* when compared with non-breeding females. They suggested that this may be due to hormonal changes or stress causing the redistribution of Hg in body tissues.

1.3.2.5. Distribution within organs

The pattern of site distribution of metals within the organism is tissue and metal specific. For example, Hg is mostly concentrated in the liver, with kidney and muscle having successively lower levels. On the contrary, the highest Cd concentrations are usually encountered in kidney due to the presence of metal binding proteins. This pattern prevails in most marine mammal concentrations (Wagemann and Muir, 1984).

Yamamoto et al. (1987) have studied the distribution of heavy metals in the whole organism of three Weddell seals (*Leptonychotes weddeli*) from the Antarctic. These authors estimated the whole metal body burden, which was calculated from the weight of the different tissues and their respective concentrations. If whole body burdens of metal are estimated for an adult Weddell seal, Zn is mostly located in muscles and in bones, Cu in muscles and liver, Hg in liver and muscles and Cd in liver and kidney. The apparent contrast between the low concentration in Hg generally measured in marine mammal muscles and the high burden encountered is due to the high muscle mass of these animals. The redistribution of Hg from highly contaminated organs such as liver or kidney through muscles seem to be a protection mechanism against Hg toxicity (Cuvin-Aralar and Furness, 1991).

Skin contains generally low Hg concentrations (Yamamoto et al., 1987). However, the skin of marine mammals is not a homogenous tissue. It consists of four distinct layers in which Hg increases progressively outwards with a concentration of 6 $\mu g.g^{-1}$ (estimation in dry weight) in the outermost layer of Arctic belugas and narwhals. During the process of moulting, the last and the underlying are shed and approximately 20% of the total Hg in the skin is lost annually in belugas and 14 % in narwhals (Wagemann et al., 1996).

1.3.3. TOXICITY OF MERCURY AND CADMIUM

As quoted above, the accumulation through the food chain is a major risk for top predators. The accumulation of chemical substances may result in toxic concentrations in organisms (secondary poisoning) even if the concentration in the environment remains below the threshold level for direct toxicity (Nendza et al., 1997). The finding of high concentrations of metals such as Cd or Hg have raised questions about their toxicity.

Reliable toxicity data for predatory marine mammals are scarce. Instead, threshold levels are often extrapolated from terrestrial species, *i.e.* interspecies correlations are assumed to hold for rats or captive seals. The validity of these extrapolations is highly questionable and can only be justified by the current lack of better data and by ethic considerations. Indeed, potential effects of toxic metals cannot be tested in free-living animals because experimental manipulations are undesirable. *In vitro* experimentations or systematic post-mortem investigations to establish the disease status of contaminated animals in a relatively large number of individuals from the same species are a complementary and valuable alternative way to understand the numerous processes involved.

1.3.3.1. Mercury toxicity

Hg exists in several interchangeable forms in the biosphere (Kaiser and Tölg, 1980), but Hg accumulation through the food web mainly occurs under an organic form, methylmercury (MeHg), as a result of its lipid solubility and preferential assimilation during zooplankton grazing (Mason et al., 1995). Above zooplankton, organic Hg is biomagnified along the food chain up to marine mammals.

Total Hg concentration is a poor indicator of toxic effects, as organic Hg compounds seem to be considerably more toxic to animals than inorganic Hg. The biological and toxicological activity of Hg depends on the form that is taken up, the route of entry in the body (skin, inhalation or ingestion), and on the extent to which Hg is absorbed (Kaiser and Tölg, 1980). Wolfe et al. (1998) have recently reviewed the toxicity of Hg on wildlife.

In mammals, methylmercury toxicity is primarily manifested as central nervous system damage including sensory and motor deficits and behavioural impairment. Animals become anorexic and lethargic. Methylmercury is easily transferred across the placenta (Wagemann et al., 1988) and thus concentrates in the foetal brain (Wolfe et al., 1998). This reproductive effect ranges from development alterations in the fœtus to fœtal death.

Methylmercury is also absorbed by the gastrointestinal tract while inorganic salts of Hg are less readily absorbed. It is mainly as the methylated form that Hg is absorbed from fish, since almost all the Hg present in fish is methylated (Svensson et al., 1992).

Experimental seal intoxication has led the animals to lethargy, weight loss and finally death (Ronald and Tessaro, 1977). Four harp seals (*Pagophilus groenlandicus*) were intoxicated with methylmercury by a daily oral intake. Two seals were fed with Hg doses of 0.25 $mg.kg^{-1}$ of body weight per day for 60 and 90 days. These two seals did not show abnormal blood concentrations but exhibited a reduction in appetite and consequent weight loss. Two others seals fed 25 $mg.kg^{-1}$ of body weight per day died on day 20 and day 26 of exposure. The measurements of blood parameters indicated toxic hepatitis, uremia and renal failure. These pathologies have been related to high accumulation of Hg in these tissues. The liver concentrations reached more than 500 $\mu g.g^{-1}$ dry weight after the death. Almost 90% of the Hg analysed in the liver was methylmercury. No detoxification mechanisms were described in this case.

However, this experimental study did not reproduce the real daily food intake of marine mammals in the wild. Nigro and Leonzio (1993) have calculated a mean daily food for small cetaceans of approximately 3 kg of fishes and cephalopods with an average Hg concentration of 0.3 $mg.kg^{-1}$ fresh weight, the mean dietary Hg intake for an adult specimen can be estimated at 0.9 mg Hg per day for the whole animal. It is quite far from the 25 $mg.kg^{-1}$ of body weight and per day administrated in gel caps to those seals. Another feature that might explain the absence of detoxification is the absence of selenium added to the food. In the wild, if fish are an source of exposure to Hg, they are also a source of selenium (Svensson et al., 1992) and in marine mammals demethylation mechanisms occur in the presence of selenium (see below). In this experimental study, the lack of additional

selenium in the diet was probably the limiting factor to detoxification.

Very few studies have tried to link metal concentrations measured in free ranging marine mammals and health status (Hÿvarinen and Sipilä, 1984; Rawson et al., 1993; 1995; Siebert et al., 1999; Bennet et al., 2001). Only one case of Hg toxicose has been reported by Helminen et al. (1968): the ringed seal suspected of Hg intoxication was from an area of heavy industrial Hg dumping.

Chronic Hg accumulation was associated with liver abnormalities observed in stranded bottlenose dolphins from the Atlantic. Large deposits of a brown pigment, identified as lipofuscin, in the portal areas of the liver were observed in the livers of nine animals with high hepatic Hg levels (> 60 µg/g fresh weight). Analytical electron microscopy carried out on these pigments demonstrated that HgSe was the predominant material (Rawson et al., 1995). Lipofuscin is believed to be derived from damaged subcellular membranes. This pigment accumulation strongly correlates with Hg concentrations. Hg would have inhibited the activity of lysosomal digestive enzymes and therefore, reduced degradation of proteins. This has led to excessive accumulation of lipofuscin within cells and finally cell death. (Rawson et al., 1993).

More recently, Siebert et al. (1999) examined the possible relationship between Hg tissue concentrations and disease in harbour porpoises from the German waters of the North and Baltic Seas. A higher Hg content has been measured in organs of the harbour porpoises from the North Sea compared to those of the Baltic Sea, indicating that Hg is a more important threat for animals of the North Sea than for the Baltic Sea. High Hg concentrations were associated with a prevalence of parasitic infection and pneumonia.

Bennet et al. (2001) have also used this indirect approach to investigate the prediction that increased exposure to toxic metals results in lowered resistance to infectious disease in harbour porpoises from the coasts of England and Wales. Mean liver concentrations of Hg, Se, Hg:Se ratio, and Zn were significantly higher in the porpoises that died of infectious diseases (parasitic, bacterial, fungal and viral pathogens such as pneumonia), compared to porpoises that died from physical trauma (most frequently entrapment in fishing gear). Liver concentrations of Pb, Cd, Cu, and chromium did not differ between the two groups.

In some cases, balances between elements seem to be more important than the absolute concentration when the possibility of toxic effects are considered. High premature birth rates have been observed and studied between 1968 and 1972 in the Californian Sea lion (*Zalophus californianus*) from the southern California Channel Island rookeries (Martin et al., 1976). These premature pups were ataxic, had difficulties in breathing and died shortly after birth. Heavy metals were analysed and compared between normal and premature pups and between their respective mothers. The results revealed that severe imbalance in the Hg:Se:Br occurred in the livers of the abnormal mothers. The absolute concentrations did not seem to be involved in this case as Hg, Se, and Br were in higher concentrations in the livers of normal mothers compared to abnormal mothers. This suggests that the Se:Hg balance is a very complex phenomenon and might be more important for general health status than absolute concentrations.

Some *in vitro* studies have also been realised to evaluate the potential hazard of Hg in marine mammals. Freeman and Sangaland (1977) demonstrated that methylmercury alters the *in vitro* synthesis of steroid hormones which play an important role in reproduction. Genetic effects of methylmercury on lymphocytes of one bottlenose dolphin (*Tursiops truncatus*) have also been evaluated *in vitro* by Betti and Nigro (1996). Lymphocytes were isolated from blood obtained from a 15 year old dolphin (Adriatic Sea). Methylmercury induces DNA single-strand breaks and cytotoxicity in a dose–dependent manner. The doses of MeHg used in this study are likely to be in the range of concentrations (between 1 and 10 µg/ml) naturally occurring in the blood of wild dolphins found in the Mediterranean Sea (Betti and Nigro, 1996). It appears that dolphin lymphocytes have a greater resistance both to the genotoxic and cytotoxic effects of MeHg when compared to human or rat cells. This feature can be interpreted as an adaptation acquired by dolphins to counter the methylmercury exposure.

1.3.3.2. Cadmium toxicity

Cd is considered as one of the most toxic metals. High dietary concentrations of Cd in humans can lead to well known heavy skeletal deformities ('itai-itai' disease), kidney lesions (mainly on

the proximal tubules) usually preceding lung damages, dysfunction of cardiovascular and hematopoietic system as well as carcinogenic, mutagenic and teratogenic effects (Förstner, 1980; Lamphère et al., 1984; Jonnalagadda and Prasada Rao, 1993). Effects of Cd on marine ecosystems have been recently reviewed (AMAP, 1998). The renal concentrations can reach levels as high as 2000 $\mu g.g^{-1}$ dry weight in some Arctic ringed seals (Dietz et al., 1998). This is much higher than the critical concentrations of approximately 800 $\mu g.g^{-1}$ dry weight (200 $\mu g.g^{-1}$ fresh weight) associated with kidney damage in mammals including humans (WHO, 1992). Moreover, following Elinder and Järup (1996), this critical concentration has been largely overestimated as Cd-induced renal dysfunctions have been displayed within order of kidney cortex concentrations of 200 $\mu g.g^{-1}$ dry weight (50 $\mu g.g^{-1}$ fresh weight). For comparison, in human adults, the renal Cd concentrations amongst non-smokers is less than 5 $\mu g.g^{-1}$ fresh weight (Pesch et al., 1989). No obvious Cd toxic effect has been registered till now in marine mammals despite the high levels encountered in several species suggesting highly efficient detoxification mechanisms (Dietz et al., 1998).

1.3.4. DETOXIFICATION MECHANISMS

The exposure of marine mammals to heavy metals has occurred throughout their evolutionary history, during which they have developed mechanisms either to control the internal concentration of certain elements or to mitigate their toxic effects. The most obvious case is the one of Hg in dolphins.
Compared to other terminal consumers like tunas or seabirds, some marine mammals accumulate much higher levels of Hg with biomagnification factors in respect to prey of 500 in dolphins compared to *e.g.* 30 in predatory fish (Leonzio, 1996). This can be explained by physiological differences not only in the involved uptake and release, but also - and sometimes mainly - in detoxification processes.

1.3.4.1. Mercury detoxification

Wagemann and Muir (1984) found Hg and selenium concentrations reaching up to 510 $\mu g.g^{-1}$ fresh weight

(approximately 2000 $\mu g.g^{-1}$ dry weight). Despite such extremely high values, the animals did not show any overt signs of Hg or selenium poisoning because the presence of the two elements together provided protection to the animal. Many studies have demonstrated the mutual antagonism between Hg and selenium (Pelletier, 1985; Cuvin-Aralar and Furness, 1991). This has become one of the strongest and most general examples of interactions between heavy metals. This phenomenon occur throughout the animal kingdom from oysters, shrimps to marine mammals and human beings. Koeman et al. (1973; 1975) first reported the strong correlation between Hg and selenium in livers of marine mammal species. A molar ratio Hg:Se of approximately 1 has been observed suggesting Hg detoxification mechanisms in presence of selenium.

Different forms of Hg coexist in the environment. Methylmercury is known to be one of the most toxic. Hg is transferred up to marine mammals in a methylated form. However, very small amounts of methylmercury are generally found in the liver of marine mammals: less than 10% of Hg is present in a methylated form in the livers of adult marine mammals. Hg also occurs in an inorganic form (Wagemann et al., 1998) which implies that a demethylation process occurs (Joiris et al., 1991). The fate of this inorganic Hg has been mainly elucidated by histological studies carried out in livers from specimens of Cuvier's beaked whale (*Ziphius cavirostris*) and bottlenose dolphins (Martoja and Viale, 1977; Martoja and Berry, 1980). These authors first observed granules composed of mercuric selenide (HgSe). Successively, similar granules were also described in the striped dolphin (*Stenella coeruleoalba*). Hg and selenium occurred as dense intracellular granules, located mainly in the liver macrophages, the Kupfer cells, and in the proximal tubules of the kidney. These granules appear as spherical or polygonal particles ranging from 15 to 80 Å (Augier et al., 1993a; Nigro and Leonzio, 1993; Nigro and Leonzio, 1996).

More recently, Rawson et al. (1995) found HgSe crystals in both the liver and respiratory system of the bottlenose dolphin and short-finned pilot whale and reported HgSe in the lung and hilar lymph nodes associated with soot particles. In both the liver and hepatic lymph nodes, these crystals were small, averaging 50Å. In the lung and hilar lymph nodes, the crystals were much larger, measuring 250-500 Å. Abundant carbon was present in the hilar nodes while only very small amounts were found in the hepatic nodes and in the liver. These findings suggest an alimentary and

a respiratory entry for Hg in cetaceans: Hg in the liver is likely to be trophically acquired, passing through the gastrointestinal tract and carried to the liver by the way of portal veins. In the liver, it may be converted into HgSe accumulating as an end product (Martoja and Berry, 1980). In animals producing large amount of HgSe, some of this may be carried to the hepatic lymph nodes and even to the spleen (Rawson et al., 1995). HgSe in the lungs and the hilar nodes appears to be closely associated with carbon suggesting an atmospheric association between these elements. Indeed, Hg and selenium pollution is largely attributed to the burning of fossil fuel or waste incinerations and these elements tend to aggregate as particles (Rawson et al., 1995). However, *in vivo* precipitation of HgSe into the surfaces of inhaled soot particles cannot be ruled out and further investigations are needed to understand this lung accumulation process.

These mercuric selenide granules seem to be the last step of a very efficient detoxification mechanism leading to high but non-toxic concentrations in the organs. The 1 molar ratio has been generally observed in marine mammals. However, the Hg:Se molar ratio found in the liver can vary within the range of 0.2 (Hansen et al., 1990) to 2.49 (Caurant et al., 1996).

Palmisano et al. (1995) have explained this variation: Hg:Se molar ratio of approximately 1 has been observed in striped dolphin livers only after a certain threshold in the total Hg concentration (approximately 100 μg $Hg.g^{-1}$ fresh weight) has been exceeded. Palmisano et al. (1995) have proposed a two-stage mechanism for the demethylation and accumulation process. At low Hg levels (first stage) the metal is retained mainly in its methylated form. At higher Hg levels (second stage) demethylation, with a concurrent accumulation of Se, seems to be the prevailing mechanism. Moreover, above the threshold only a small fraction of Hg is present in a labile form as Hg^{++} and $MeHg^{+}$ bound to the cysteine residue. These authors have determined that 63% of the total Hg analysed in the liver of one dolphin is involved in the formation of a very insoluble selenocompounds, certainly present as HgSe (tiemannite) but in addition, Hg can be involved in the formation of other seleno-compounds as Hg-selenoproteins.

This hypothesis of a threshold has also been suggested in other studies dealing with Hg speciation (Sanpera et al., 1993; Caurant et al., 1996). Sanpera et al. (1993) have found no decrease in the organic Hg fraction with increasing total Hg concentration in the livers of fin whales (*Balaenoptera physalus*).

On the contrary, Caurant et al.(1996) found in livers of longfinned pilot whales a decreasing correlation between organic Hg and total Hg. Organic Hg was lower than 2% when the total Hg concentration was higher than 100 $\mu g.g^{-1}$ fresh weight (400 $\mu g.g^{-1}$ dry weight). Only a small fraction (<1%) of total Hg is bound to heat-stable compounds (which include the metallothioneins). These heat-stable proteins are able to bind bivalent metals and are believed to participate in heavy metal detoxification processes (see paragraph 1.4.). More than 90% of the Hg was in the insoluble fraction except for two animals. In these two individuals, insoluble Hg in the liver was < 90% and 14% and 35% of Hg were bound to metallothioneins. These individuals exhibited total Hg concentrations <50 $\mu g.g^{-1}$ fresh weight (200 $\mu g.g^{-1}$ dry weight) in the livers. When total Hg was higher than this value, the percentage bound to metallothioneins was low always <1% whatever the total Hg concentration (Caurant et al., 1996).

In some species, Hg concentrations can be low during the entire life span. Fin whales feed at the bottom of the trophic web (Sanpera et al., 1993) and their Hg levels stay relatively low. In their livers, the mean ratio of organic/total Hg is about 40%. This result seems very high compared to other data (see table 1.2). The authors suggest that demethylation is carried out at a constant rate, probably because concentrations remain low throughout their life span, between 50 and 500 $\mu g.g^{-1}$ dry weight (Sanpera et al., 1993). It seems in this case that the specific threshold has not been reached and so high percentages of organic Hg are observed in the livers.

Young marine mammals can display a high percentage of methylmercury (table 1.2) suggesting that they do not reach a specific threshold (Palmisano et al., 1995). A second hypothesis is that young individuals are still unable to demethylate Hg efficiently (Caurant et al., 1996).

Table 1.2. Comparison of mean percentage (%) methylmercury to total mercury in suckling marine mammals compared to adults.

Species	Pups	Adults	Sources
Ringed seal *Pagophilus groenlandicus*	70	14	Wagemman et al., 1988
Striped dolphin *Stenella coeruleoalba*	45	2.5	Itano et al., 1984
Long-finned pilot whale *Globicephala melas*	55	17	Caurant et al., 1996

1.3.4.2. Cadmium detoxification

As quoted above, the concentrations of Cd in kidneys of marine mammals can reach levels more than twice the critical concentrations of approximately 800 $\mu g.g^{-1}$ dry weight (200 µg/g fresh weight) associated with kidney damage in terrestrial mammals including humans (WHO, 1992). This raises questions about the health status of such heavily Cd contaminated marine mammals. Dietz et al. (1998) have compared low and high Cd contaminated kidneys from ringed seals from Northwest Greenland in an attempt to do macroscopic and light microscopic examinations. No differences in renal morphology could be observed between experimental groups. These investigations indicate that marine mammals appear to be able to maintain considerable concentrations of Cd without showing renal damage. Dietz et al. (1998) have therefore postulated that ringed seals are adapted to the naturally high Cd levels of the Greenland Arctic regions.

Metallothioneins

Marine mammals might mitigate the toxic effects of Cd through binding to small specific proteins called metallothioneins (MTs) (reviewed by Das et al., 2000). The presence of these low-molecular weight proteins has been demonstrated in many organisms ranging from blue green algae to sperm whales (Kägi, 1991; Holsbeek et al., 1998).

MTs are involved in general essential metal homeostasis and non-essential metal detoxification. The role of metallothioneins in marine mammals has been recently reviewed (Das et al., 2000) and constitutes the sixth part of the general introduction of this work (see section 1.4. for further information on metallothioneins).

Cadmium spherocrystals

Cd-containing granules have been observed in the kidney of two white-sided dolphins (Gallien et al., 2001). These two individuals with high Cd concentrations exhibited electron dense mineral concretions of diameters up to 300 nm in the basal membranes of the proximal tubule. These spherocrystals are made up numerous strata of mineral deposits of calcium, phosphorus and Cd. Cd has been detected with a molar ratio of Ca:Cd of 10:1 in the middle of these concretions.
The occurrence of metal-containing granules is well documented in invertebrates (Simkiss, 1976) but this is the first report of granules containing Cd in wild vertebrates. In these marine mammals exhibiting high levels of Cd, these granules could constitute a means of immobilisation and detoxication.

1.3.5. LIMITS TO DETOXIFICATION AND CONCLUSIONS

Remarkable tolerance of marine mammals to heavy metals has been suggested through several detoxification processes such as tiemmanite storage and binding to metallothioneins, but is there a limit to the detoxification process and if so, what is the actual hazard of heavy metals?

The ratios between different metals appear more important than their absolute concentrations (Martin et al., 1976; Becker et al., 1995). Pups are more affected by these metals as they exhibit a higher methylmercury ratio compared to total Hg due to their poorly efficient detoxification mechanism (Wagemann et al., 1988). Moreover, a depressed molar ratio of Br:Hg:Se in premature pups of California sea lions was suggested to be a main death factor (Martin et al., 1976).

Caurant et al. (1996) have proposed that detoxification of Hg could be limited in lactating longfinned pilot whales . Indeed, compared to other females, Hg concentrations were much higher in lactating females, while selenium concentrations were lower. Squid is the major food item of pilot whales, but a greater quantity and variety of fish species have been observed in the diet of lactating females. The authors suggested that the energy value of fish is higher than squid and a higher consumption of fish would cover the increasing need of energy to produce milk. The percentage of selenites (inorganic form of selenium) and the inorganic forms of Hg seem to be higher in squids than in fish muscles where methylated Hg is dominant. Most of selenium found in fish occurs in an organic form, which is less efficient than selenites in preventing the toxicity of Hg. The different diet of lactating females could induce both higher levels of Hg and a lower efficiency of the detoxification process (Caurant et al., 1996).

Binding of Cd to metallothioneins could also be a limiting factor to detoxification (Bouquegneau et al., 1997). In debilitated sperm whales stranded on the Belgian coast, only a small amount of Cd was bound to metallothioneins (Bouquegneau et al., 1997; Holsbeek et al., 1999). As quoted above, this could explain the observed debilitation which could have favoured their stranding. Precipitation of Cd under a granule form could also lead to some toxic effects (Gallien et al., 2001). The authors underlined the fact that lesions could be associated to these granules especially in

older animals exhibiting high Cd concentrations and if so, detoxification processes could lead to some toxic effects.

We have to keep in mind that any detoxification process has a cost for the cell or the organism involved and might have a threshold. This threshold can not be fully defined in terms of tissue concentration because of the number of parameters that can interact to limit physiological pathways that lead to detoxification. For example, gender and hormonal activity can modulate the synthesis of metallothioneins (Blazka and Shaikh, 1991). Moreover, detoxification processes can lead to the formation of compounds which could have toxic effects. The accumulation and degradation of Cd-metallothionein complex (CdMT) in the renal tubular epithelial cells can induce nephrotoxicity in mice counteracted by Zn which has a protective effect against this CdMT-induced nephrotoxicity (Liu et al., 1996; Tang et al., 1998). As a result of their physiological function in the homeostasis of essential metals, metallothioneins could be involved in many cellular pathways. Thus, they could modulate physiological processes as an indirect effect of heavy metal exposure. For example, metallothioneins have been demonstrated as potential modulators of some parameters of the immune response (Leibbrandt et al., 1994; Borghesi et al., 1996) . Detoxification pathways could therefore lead to more subtle toxic effects underlying the complexity of the toxic effects of heavy metals.

To conclude, the actual toxic effects of heavy metals on marine mammals remain unclear. Are they responsible - even in part - for the decline of some marine mammal species? As quoted above, that decline is obviously multifactorial: past overfishing, present increasing human activities, accumulation of pollutants among which heavy metals can not be neglected. The role of marine mammals on the whole marine ecosystem is still poorly understood: their contribution in the recycling of nutrients is not very important, but their part in structuring marine communities and in modifying benthic habitats are more and more evident (Bowen, 1997). Marine mammals presently consume at least three times greater quantity of prey than do human fisheries, but that could be low compared to their ecological role, still poorly understood. Some species compete with fisheries, while others obviously do not, and yet others partly compete but could be useful to fisheries by regulating the development of non-commercial species, thereby limiting excessive competition with commercial ones (Bouquegneau et al., 1997a). It is our belief that

marine mammals deserve their place in the oceans and are worth being protected.

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1.4. METALLOTHIONEINS IN MARINE MAMMALS

(After Das K., Debacker V., Bouquegneau J.M. 2000. Metallothioneins in marine mammals. Cellular and Molecular Biology 46 (2): 283-294

1.4.1. FOREWORD

Many areas have emerged in metallothionein (MTs) research since their were first discovered (Margoshes and Vallee, 1957), but their natural function remains elusive (Palmiter, 1998). Previous studies on structure, function, and molecular regulation have established a central role for these molecules in the homeostatic regulation of essential metals such as zinc (Zn) and copper (Cu). Thus, it is not surprising that MTs have been detected in both prokaryotes and eukaryotes including marine mammals (reviewed by Roesijadi, 1992;1996). Indeed, the use of metals as cofactors in biochemical reactions and their toxicity associated with their affinity for S, N, and O (the predominant ligands in biomolecular structures), represent dual aspects of metal-biological interactions. Therefore, mechanisms that regulate the intracellular availability of essential metals and protect against inappropriate and potentially deleterious intracellular interactions are primordial for efficient biochemical function. MTs work as Zn or Cu donors to other metalloproteins. They are induced by and bind excesses of these metals. Another function postulated for MTs is the detoxification of non-essential metals like cadmium (Cd) and mercury (Hg). As a result of its capacity to bind cations, MTs are able to bind non-essential metals such as Cd^{2+} Ag^{+}, Hg^{2+} and Pb^{2+} and, in this way, reduce the bioavailability of these toxic metals (reviewed by Webb, 1987 and Roesijadi, 1992).

It has been suggested that Cd toxicity occurs when available MTs are insufficient to bind all the Cd. Recent experiments with mice genetically deprived of MTs due to the loss of functional MT I and II genes (coding for the 2 main isoforms of MTs involved in the detoxification process) confirm the protective role of these proteins against cellular damages from metals such as Cd or

inorganic Hg (Satoh et al., 1997; Klassen and Liu, 1998). This leads to consider that these proteins prevent organisms from toxic hazards that could occur following the high exposure to Cd and Hg. Recently, Klaassen et al. (1999) reviewed the protective action of MTs against Cd toxicity.
So far, there is little data on MTs in marine mammals. Nevertheless, in the framework of heavy metal ecotoxicology, marine mammals appear as a choice material, since high levels of Hg and Cd can be naturally encountered in these animals. While plankton-eating Mysticetes are generally weakly contaminated by heavy metals, fish-eating and squid-eating Odontocetes and Pinnipeds are heavily contaminated by Hg and Cd respectively (Bouquegneau and Joiris, 1992). Heavy metals in marine mammals have been recently reviewed by Das et al. (2000).

A maximal Hg concentration of about 13000 µg/g dry weight has been reported in the liver of one bottlenose dolphin stranded on the Italian coast (Leonzio et al., 1992). Such high levels of Hg without overt evidence of deleterious effects may only occur if Hg is detoxified. Koeman et al. (1973; 1975) have first reported a strong correlation between Hg and selenium in livers of marine mammals. A molar ratio Hg:Se of approximately 1 has been observed suggesting Hg detoxification mechanisms in presence of selenium. The fate of Hg has been mainly elucidated by histological studies carried out in livers from different marine mammals species as Cuvier's beaked whale *Ziphius cavirostris* and bottlenose dolphins *Tursiops truncatus* (Martoja and Viale, 1977; Martoja and Berry, 1980; Nigro and Leonzio, 1996). These authors have observed mercuric selenide granules (HgSe) located mainly in the liver macrophages, the Kupffer cells, and in the proximal tubules of the kidney. The transformation of assimilated Hg in tiemmanite (HgSe) appears to be the last step of detoxification leading to formation of inert and non-toxic Hg compounds.

In the same way, Cd renal concentrations can reach levels as high as 2000 $\mu g.g^{-1}$ dry weight in some Arctic ringed seals without any pathological effects (Dietz et al., 1998). This is much higher than the critical concentrations of approximately 800 $\mu g.g^{-1}$ dry weight (200 $\mu g.g^{-1}$ wet weight) associated with kidney damage in mammals (WHO, 1992). Moreover, following Elinder and Järup (1996), this critical concentration has been largely overestimated as Cd-induced renal dysfunctions have been observed at kidney cortex concentrations of 200 $\mu g.g^{-1}$ dry weight (50 $\mu g.g^{-1}$ wet weight). For comparison, in human adults, the renal Cd

concentrations amongst non-smokers is about 1 $\mu g.g^{-1}$ wet weight (Pesch et al., 1989). These investigations indicate that marine mammals are able to maintain considerable concentrations of Cd without showing renal damages. It has been postulated that marine mammals mitigate the toxic effects of Cd through binding to MTs. So far, MTs and metallothionein-like proteins (MTLP) have been described in 10 marine mammal species (table 1.3) with concentrations ranging from 58 to 710 $\mu g.g^{-1}$ and 140 to 1200 $\mu g.g^{-1}$ fw in the liver and kidneys respectively.

1.4.2. CADMIUM BINDING

As quoted above, the renal concentrations of Cd in marine mammals can reach levels much higher than the critical concentrations associated with kidney damage in terrestrial mammals. The question has therefore been raised about animals so heavily contaminated with Cd. Dietz et al. (1998) have compared low and high concentrations of Cd in the kidneys of ringed seals (*Phoca hispida*) from Northwest Greenland in an attempt to do macroscopic and light microscopic examinations. No differences in renal morphology could be observed between experimental groups. These investigations indicate that marine mammals appeared able to maintain considerable concentrations of Cd without showing renal damage. They therefore postulated that ringed seals were adapted to the naturally high Cd levels of the Greenland Arctic regions.

The role of MTs in binding Cd present in the tissues may vary widely between different species as well as between different individuals from the same species (table 2). This leads to more or less important spillage of Cd to other metalloproteins. It is interesting to note that the low values of 5, 17 and 18% have been measured in the liver of three highly debilitated sperm whales found stranded on the Belgian coast (Bouquegneau et al., 1997b; Holsbeek et al., 1999). Moreover, a spillage of Cd from MTs to other soluble components has been observed: 34 to 38 % of the total Cd is bound to soluble proteins other than MTs (calculated from Holsbeek et al., 1999). This suggests that Cd was not in a detoxified form, either on MT or in lysosomes. These animals were seriously debilitated as indicated by their reduced blubber thickness and body weight (Jauniaux et al., 1998). Cd which is known to induce debilitation in mammals can be considered as one of the factors responsible for the condition of

these animals, which in addition to stress and starvation, could have resulted in their stranding (Bouquegneau et al., 1997b).

Table 1.3. Metallothionein and metallothionein-like protein detection and quantification (metal-free protein) in marine mammals (data about MT concentrations are either single value or in range, or mean ± standard deviation). Metallothionein concentrations are estimated assuming 7 gram-atoms (Cu, Zn, Cd, Hg) per mole and a molecular weight of 7 kDa

	Species	Geographic location	Tissue	concentration ($\mu g.g^{-1}$ ww)	Isoforms detected	Method	References
Pinnipeds	**California sea lion** *Zalophus californianus californianus*	Oregon (USA)	kidney	**140** (n=1)	MT-1 MT-2	Sephadex G-75 or Sephadex G-200 Whatman DE 32-column	Lee et al., 1977
	Ribbon seal *Histriophoca fasciata*	Japan	liver	nd	MT-1 MT-2	Sephadex G-75 HPLC-AAS DEAE-3SW column	Mochizuki et al., 1985
	Harbour seal *Phoca vitulina*	Japan	liver	nd	MT-1 MT-2	Sephadex G-75 HPLC-AAS DEAE-3SW column	Mochizuki et al., 1985
		Japan	liver	**240** ± 139 (n=15)	nd	Radioimmunoassay	Tohyama et al., 1986
			kidney	**343** ± 219 (n=15)			
	Grey seal *Halichoerus grypus*	Canada	liver	**70** (n=1)	nd	Sephadex G-75 followed by G-50	Olafson and Thompson, 1974
	Pacific fur seal *Callorhinum urisnum*	Canada	liver	**90** (n=1)	nd	G-75 followed by G-50	Olafson and Thompson, 1974
	Pilot whale *Globicephala melas*	Faroe Islands	liver	**167** ± 36 (n=7; july 1986) **592** ± 200 (n=7; november 1986)	nd	Polarography	Caurant et al., 1996; Amiard Triquet and Caurant, 1997
			kidney	**751** ± 213			
Odontocetes	**Striped dolphin** *Stenella coeruleoalba*	Nothwestern Pacific	kidney	**314**[1] (n=1)	MT-1 MT-2	Sephadex G-75 Sephadex G25 DEAE Sephadex-A25 HPLC on GS-320 column Amino acid characterisation and primary structure	Kwohn et al., 1986; 1988
	Narwhal *Monodon monoceros*	Arctic	liver	**710**[1] (n=1)	nd	Sephadex G-75, Sephadex G-50 Polyacrylamide gel electrophoresis DEAE Sephadex A25	Wagemann et al., 1984 Wagemann and Hobden, 1986
			kidney	**1200**[1] (n=1)			
	Harbour porpoise *Phocoena phocoena*	North Sea	liver	nd	nd	AcA-54	Antoine et al., 1992
	Sperm whale *Physeter macrocephalus*	USA	liver	nd	nd	Sephadex G-75 and DEAE Sephadex gel	Ridlington et al., 1981
		North Sea	liver	**58** [1] (n=3)	nd	AcA-54	Bouquegneau et al., 1997; Holsbeek et al., 1999
			kidney	**468-951** [1] (n=2)			

However, high levels of Cd with low binding to MTs have been reported in healthy animals. Amiard-Triquet and Caurant (1997) have reported that 51% of total Cd were bound to MTs in the livers of pilot whales caught in July 1986 whereas individuals caught in November from the same year displayed only 6%. Moreover, individuals caught in winter have low plasma Cd levels. According to these authors, Cd in the plasma resulted from a recent Cd contamination. These results are in agreement with the seasonal availability of preys: squids which are known to concentrate Cd in their tissues, are more abundant in summer. So it seems that only recently assimilated Cd is bound to MTs, the rest being stored in the insoluble fraction (Amiard-Triquet and Caurant 1997). In contrast to pilot whales, in the narwhal, more than 70 % of the cytosolic Cd is located in the MT fraction of the liver, indicating low spillage of Cd to other metalloproteins (Wagemann et al., 1984). This could be an adaptation of this Arctic species to the high Cd concentrations present in its environment. However, more data on Cd speciation in marine mammals are needed to get a better comprehension of the precise role of MTs in detoxification processes.

1.4.3. MERCURY BINDING

Hg also has a strong affinity for MTs and high levels of this metal can be associated with elevated levels of Cd in marine mammal tissues (Caurant et al., 1996). However, studies conducted on dolphins (Kwohn et al., 1986), California sea lions (Lee et al., 1977), pilot whales (Caurant et al., 1996), narwhals (Wagemann et al., 1984) and sperm whales (Bouquegneau et al., 1997b; Holsbeek et al., 1999) demonstrated that only a small part of the total Hg was bound to MTs (table 1.5). A significant amount of Hg measured in the cytosol of a narwhal liver was found to be associated with the high molecular weight fractions (Wagemann et al., 1984). According to these authors, such a spill over of Hg to the high molecular weight components would be a normal occurrence in marine mammals and is not related to the saturation of MTs.

Even though there is a high affinity of Hg for MTs, most of the metal is bound to components other than MTs. These results are quite different from those deriving from studies carried out on terrestrial mammals, in which Hg is shown to be particularly bound to MTs (Whanger and Deagen, 1983). This striking

difference between terrestrial and marine mammals is mainly due to differences in Hg speciation in the diet. In the marine environment, almost all the Hg present in fish is methylated (Svensson et al., 1992). Methylmercury, whose affinity for MTs is low, cannot be detoxified by this process. In marine mammals, it has been shown that the relative MeHg levels decreased from 100% (of the total Hg level) in juveniles to only 2 or 3 % in the liver of adults (Joiris et al., 1991). This reflects the existence of a slow mineralisation process without formation of free Hg^{++} ions which can bind to MTs.

Table 1.4. Cd speciation in the tissue, the cytosolic fraction and metallothioneins (Cd concentrations are estimated in $\mu g.g^{-1}$ dry weight; nd: not determined, dl: detection limit).

Species	Tissue	n	Total Cd ($\mu g.g^{-1}$ dw)	% of Cd in the cytosolic fraction	% cytosolic Cd on MTs	References
Striped dolphin *Stenella coeruleoalba*	kidney	4	87	58	98	Kwohn et al., 1986
California sea lion *Zalophus californianus*	kidney	1	37	68	nd	Lee et al., 1977; Ridlington et al., 1981
		5	65 ± 30	63	71	
	liver	1	<dl	<dl	<dl	
		5	11 ± 7	60	55	
Pilot whale *Globicephala melas*	kidney	7 gestating females	548 ± 164	nd	54 ± 6	Amiard-Triquet and Caurant, 1997
		5 foetuses	1 ± 0.8		44 ± 41	
	liver	7 gestating females	312 ± 124		51 ± 20	
		5 foetuses	0.6 ± 0.7		25 ± 34	
Sperm whale *Physeter macrocephalus*	liver	1	50	92	100	Ridlington et al., 1981
		3	64	53	18	Bouquegneau et al., 1997 Holsbeek et al., 1999
			71	55	17	
			103	39	5	
	kidney	2	225	81	21	
			316	85	66	
Narwhal *Monodon monoceros*	kidney	1	332	92	72	Wagemann et al., 1984; Wagemann and Hobden, 1986
	liver	1	176	88	77	

The observed high percentage of Hg bound to the insoluble fraction of the liver results from the formation of tiemannite (HgSe) These dense intracellular granules have been observed in the liver macrophages and Kuppfer cells, the proximal tubules of the kidney, the lung and hilar lymph nodes (Martoja and Viale,

1977; Martoja and Berry, 1980; Nigro and Leonzio, 1996; Augier et al., 1993; Rawson et al., 1995). By this way, in marine mammals, selenium plays a key role in methylmercury detoxification processes, and hence MTs would play a minor role, probably limited to the detoxification of Hg^{2+}.

Table 1.5. Distribution of mercury on renal and hepatic metallothioneins

Species	Tissue	Total Hg ($\mu g\ g^{-1}$ fw)	% Hg insoluble fraction	% Hg associated with MTs	References
Pilot whale *Globicephala melas*	kidney (n=7)	6	79	7	Caurant et al., 1996
California Sea lion *Zalophus californianus*	kidney (n=5)	10	54	22	Lee et al., 1977
	liver (n=5)	61	93	2.6	
Narwhal *Monodon monoceros*	liver (n=1)	9	88	5	Wagemann et al., 1984
	kidney (n=1)	2	73	10	
Spem whale *Physeter macrocephalus*	liver (n=3)	2	85	<1	Bouquegneau et al., 1997; Holsbeek et al., 1999
		15	95	<0.3	
		15	84	<3	
	kidney (n=2)	2	72	<2	
		5	70	<6	
Striped dolphin *Stenella coeruleoalba*	kidney (n=4)	16	83	6	Kwohn et al., 1986

1.4.4. STRUCTURE AND CHARACTERISATION

Comparative sequence studies of MTs from different species and organs have revealed remarkable similarities among mammalian species (see Binz and Kagï, 1999). About 56% of the 61 amino acid residues are conserved in mammals, among them all the 20 cysteine and nearly all lysine and arginine residues (Kojima et al., 1999).

Characterisation of marine mammal metalloproteins were first attempted on sea lion and sperm whale kidney and liver (Ridlington et al., 1981). Metal binding proteins isolated from sperm whale liver eluted in a manner similar to rat MTs, but the amino acid analyses yielded only 12% cysteine residues whereas

all mammalian MTs contain approximately 30%. According to the authors, this protein was not likely a metallothionein but rather a type of Cu-chelatin. Similar conclusions were drawn for sea lion liver and kidney metal binding proteins as the amino acid analysis for the different metal-binding fractions contained 2 to 15% cysteine residues. It must be noted however, that general re-examination of Cu chelatin has resulted in its designation as a MT (Winge et al., 1981 quoted by Roesijadi, 1992).

However, results reported by Ridlington et al. (1981) are inconsistent with other marine mammal MT characterisation. Indeed, striped dolphin MT I and II isolation and characterisation have been performed (Kwohn et al., 1986; 1988). According to these authors, 61 amino acid residues are present per mole of each MT, including 20-21 cysteine residues (table 1.6). The absence of aromatic amino acids agrees with the lack of absorbance at 280 nm. MT amino acid composition of striped dolphin compared with rabbit for both isoforms is quite similar. According to Kwohn et al. (1988), 89% of the MT II amino acid sequence is conserved between rabbit and dolphin. Neither valine nor leucine were detected in rabbit both isoforms, which suggests microheterogeneity between the two species.

This discrepancy observed between the two characterisations of marine mammal MTs might be explained, at least in part, by technical evolution. Indeed, Roesijadi (1992) has underlined early technical difficulties associated with purifications of MTs. Studies that have attempted to characterise metal-binding proteins have often been influenced by the presence of impurities or the isolation of proteins onto which metals could be redistributed during sample preparation.

Kwohn et al. (1986) have detected two isoforms in the dolphin kidney identified as MTs I and II. According to its absorbance at 254 nm, the MT II isoform is much more abundant than MT I with a ratio 1/16 while, for example, it is *e.g.* only 1/6.2 in equine tissues (Kojima et al., 1976 quoted by Kwohn et al., 1986). Most of the metals are therefore bound to MT II except Cu which is preferentially bound to MT I (Kwohn et al., 1986). The same tendency is also observed for horse renal MTs (Kojima et al., 1976 quoted by Kwohn et al., 1986) indicating different cellular functions for each renal MT of striped dolphins. However, it is worth noticing that Wagemann and Hobden (1986) reported MT I as the major form of MTs in the liver and kidney from a narwhal. The functional significance of multiple isoforms of MTs has yet to

be demonstrated for any mammalian species. The different roles of MTs in the regulation of metals are probably critical to cell functions but these have yet to be studied in any marine mammal. It is clear, however, that understanding the MT function will need to consider the functionality and structure of each MT isoform.

Table 1.6. Amino acid composition of striped dolphin renal MT I and MT II (after Kwohn et al., 1986) and rabbit hepatic MT I and MT II (after Nordberg et al., 1972) *: calculated as metal free molecular weight

Amino acid	Dolphin MT I	Dolphin MT II	Rabbit MT I	Rabbit MT II
Asp	3	3	5	5
Thr	3	2	3	4
Ser	7	8	7	9
Glu	2	2	1	3
Gly	6	6	4	4
Ala	6	6	8	8
Cys	21	20	15	19
Val	1	1	/	/
Met	1	1	1	1
Ile	1	1	1	1
Leu	1	/	/	/
Phe	/	/	/	/
Lys	6	8	7	9
Arg	/	/	/	/
Pro	3	3	5	3
Tyr	/	/	/	/
His	/	/	/	/
Total	**61**	**61**	**57**	**66**
Molecular weight *	**5986**	**6013**	**5608**	**6616**

1.4.5. FACTORS MODULATING METALLOTHIONEIN CONCENTRATIONS

The range of MT concentrations in marine mammal kidneys and liver is widely extended (from 58 to 1200 $\mu g.g^{-1}$ fw). Their concentrations appear to be high compared with those in rats. Indeed, hepatic levels in normal rats are about 1 $\mu g.g^{-1}$ while it can reach more than 200 $\mu g.g^{-1}$ in Cd-induced animals (Eaton and Toal, 1982). Maximum concentrations encountered in marine mammals are much higher (table 1.1). However, the modulation of MT concentrations in marine mammals is poorly understood due to a lack of extensive quantifications. In most cases, the factors influencing MT concentrations in the wild are assumed after experimental studies on other mammals. Till now, only two

studies have attempted to correlate MTs with ecological or ecotoxicological factors (Tohyama et al., 1986; Amiard-Triquet and Caurant, 1997). According to these authors, the MT concentrations depend more on particular tissues, the heavy metal concentration, the age and the diet.

1.4.5.1. Tissue

MT concentrations are always higher in the kidneys than in the livers (table 1.7). Few data are available to allow for comparison, but MT level in the kidney appears to be 1.2 to 1.6 fold higher than in the liver (table 1.5) except for sperm whales stranded on the Belgian coast in which the ratio may be related with the debilitated status of these animals.

Table 1.7. Comparison of the ratio of MT concentrations in the kidney (K) and liver (L)

Species	n	Sampling status	Ratio $K.L^{-1}$	Reference
Pilot whale *Globicephala melas*	7	by-caught	1.2	Amiard-Triquet and Caurant, 1997
Harbour seal *Phoca vitulina*	15	by-caught	1.4	Tohyama et al., 1986
Narwhal *Monodon monoceros*	1	by-caught	1.6	Wagemann et al., 1984
Sperm whale *Physeter macrocephalus*	2	stranded	8.7-12.4	Holsbeek et al., 1999

1.4.5.2. Metal level

A study performed on harbour seals (*Phoca vitulina*) caught on Japanese coasts show that MT concentrations are significantly correlated with the level of Cd and Zn in the livers and with the level of Cd, Zn, and inorganic Hg in the kidneys (Tohyama et al., 1986). These authors suggest that inorganic Hg and Cd could be sequestered in MT and that Hg toxicity could also be lessened by this protein.

1.4.5.3. Age

Tohyama et al. (1986) have pointed out that higher MT levels have been quantified in the liver and kidney of a seal pup as compared to adults. Neonates of various mammalian species are known to have relatively high levels of MTs with associated Zn and Cu in the liver (Bakka and Webb, 1981, quoted by Tohyama et al., 1986). Amiard-Triquet and Caurant (1997) have quantified hepatic MTs in gestating female pilot whales and their foetuses. MT mean concentrations were always lower in the foetus than in mother's livers (mean: 73 $\mu g.g^{-1}$ and 167 $\mu g.g^{-1}$ respectively). However, the ratio MT/ total proteins remained unchanged. In harbour seals, Tohyama et al. (1986) found a correlation between MT levels and age. The observed age-dependant changes in renal and hepatic MTs are associated with Cd accumulation as it has been shown recently in human tissue (Yoshida et al., 1998).

1.4.5.4. Diet

As quoted above, Caurant *et al.* (1996) and Amiard-Triquet and Caurant (1997) have compared metallothionein-like protein (MTLP) concentrations between pilot whales caught in July and November 1986 (fig.1.7).

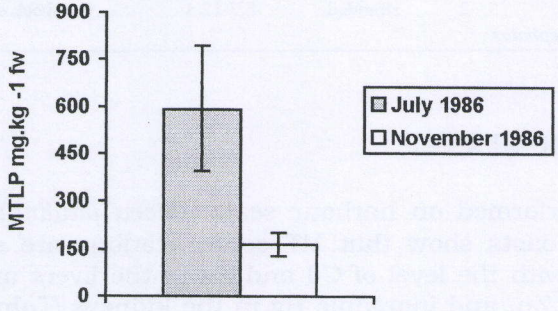

Figure 1.7. Mean concentrations and standard deviations of metallothionein-like proteins (MTLP) calculated in the livers of pilot whales (*Globicephala melas*) caught in the Faroe Islands (data from Caurant et al., 1996; Amiard-Triquet and Caurant, 1997)

MT mean concentrations in the livers of pilot whales caught in the summer 1986 are more than three fold higher than those encountered in individuals caught in winter, while Cd concentrations are similar for both groups. Squid is the major food item in pilot whales and is considered as a significant source of Cd for several predators (Das et al., 1999_b). The lower level of MTs in whales caught in November could be related to lower Cd assimilation in winter. Indeed, the summer diet of pilot whales consists mainly in cephalopods whereas the winter diet is characterised by a higher fish input (Caurant et al., 1996 ; Amiard-Triquet and Caurant, 1997). According to the authors, the elevated MT concentrations would reflect an induction by a recent Cd assimilation. The metal could be sequestered later under another detoxified form (*e.g.* precipitated within lysosomes).

1.4.6. MTs AS BIOCHEMICAL INDICATORS OF METAL EXPOSURE AND TOXICITY

Soon after their discovery in aquatic species, the induction of MT and the analyses of its capacity to bind metals were proposed as candidates for biochemical monitoring of metal pollution in the aquatic environment (Roesijadi, 1996). Heavy metal analysis in the tissues reflects the level of contamination of a population but not its response to metal exposure, as these can be detoxified, at least in part, through the binding to MTs. The induction of MTs by non-essential metals such as Cd has been considered as an asset in biomonotoring studies. Moreover, with current methodologies, MT induction can be measured at several levels: increase in metal content in the MT pool , increase in MT and increase in MT mRNA (*e.g.* Suzuki, 1992; Caurant et al., 1997; Tom et al., 1998). Each reflects a different level of cellular regulation and function, and provides complementary information. Arguments against application of MT analysis in environmental studies resides mainly in our current lack of detailed understanding of basal MT function and its relationship to induction by metals (Roesijadi, 1992 ; Cosson and Amiard, 1998). Through forty years of MT research, the different interacting processes appear numerous and complex. Cosson and Amiard (1998) have recently reviewed the utilisation of MTs as potential biomarkers of metal contamination in aquatic animals including marine mammals and have underlined the difficulty to

correlate metal bioaccumulation and increased MT level. Thus proposal for the use of MTs as biochemical indicators of metal pollution in marine mammals merits continued considerations, as further investigations are needed to understand the processes involved in detoxification.

1.4.7. LIMITS TO DETOXIFICATION

Drawing conclusions concerning the potential role of MTs in marine mammals is not an easy task due to an obvious lack of recent published studies and extrapolation from other mammals are often advanced. Concerning potential role of these proteins in Cd detoxification, opinions are mitigated. The rates of MT synthesis can become limiting as exposure concentrations increase. The related decreasing metal binding to MTs could result in the spillage of metals to other structures that would include target sites for metal toxicity (Roesijadi, 1992). Any detoxification process has a cost for the cell or the organism involved and might have a limit. This threshold cannot be fully defined in terms of tissue metal or MT concentrations because of the number of parameters that can interact to limit physiological pathways that lead to detoxification. For example, gender and hormonal activity can modulate the synthesis of MTs (Blazka and Shaikh, 1991). Moreover, binding to MTs might not be a final step, and the formation of secondary components with toxic effects have been demonstrated in other mammals. The accumulation and degradation of Cd-metallothionein complex (CdMT) in the renal tubular epithelial cells can induce nephrotoxicity in mice counteracted by Zn which has a protective effect against this CdMT-induced nephrotoxicity (Liu et al., 1996; Tang et al., 1998). As a result of their physiological function in the homeostasis of essential metals, MTs could be involved in many cellular pathways. Thus, they could modulate physiological processes as an indirect effect of heavy metal exposure. For example, MTs have been demonstrated as potential modulators of some parameters of the immune response (Leibbrandt et al., 1994; Borghesi et al., 1996). Detoxification pathways could therefore lead to more subtle toxic effects underlying the complexity to approach toxic effects of heavy metals.

1.4.8. CONCLUSIONS

Compared with other animals, liver and kidneys of marine mammals display high MT concentrations, related with their high levels of contamination by heavy metals (mainly Cd and Hg). These high heavy metal concentrations result from both their homeothermy (which requires large food consumption) and their position at the top of marine food webs. Data about MTs remain scarce and, until now, discussion is only possible about their potential role in the detoxification of Hg and Cd. Obviously, MTs play a minor role (if any, when considering methylmercury) in the binding and detoxification of Hg by marine mammals. On the contrary, close interactions occur between Cd and MT dynamics. Cytosolic MTs appear as a potential short term way of detoxification of Cd accumulated from diet. Long-term detoxification however would imply a sequestration of the metal under a precipitated form (e.g. in lysosomes). So many parameters are likely to modulate the MT concentration in marine mammals tissues that its use as a biomarker of heavy metals pollution in the marine environment remains debatable.

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1.5. STABLE ISOTOPE ECOLOGY

The use of stable isotopes has recently evolved to a powerful tool to study element cycles and various biological, physical, and chemical processes in research areas as diverse as archaeology, medicine, ecology, astronomy and hydrology. This paragraph is intended to provide an introduction to stable isotopes of carbon and nitrogen as these isotopes are used extensively throughout further chapters.

1.5.1. BACKGROUND

Most elements of biological interest have two or more stable isotopes, which differ only in the number of neutrons, resulting in different atomic masses. Therefore, different stable isotopes of the same element do not react in the same manner in fundamental kinetic processes. This phenomenon is called fractionation, which is a function of slight variations in the physical and chemical properties of the isotopes and therefore occurs in proportion to the differences in the isotopic masses. Each fractionation will result in a modification of the original ratio between the isotopic species concerned (Ehleringer and Rundel, 1989). Fractionation processes of ^{13}C and ^{15}N have been intensively used to delineate trophic relationship in different food webs (Gannes et al., 1998; Hobson, 1999; Kelly, 2000).

Carbon have two stable isotopes with respective different proportions in nature (table 1.8). Small variations in these abundances occur and these can be accurately determined with stable isotope spectrometers.

Isotopic composition is measured by determining the ratios of the two stable isotopes present in the sample. As the natural variations in the relative abundance of "light" and "heavy" isotopes of an element are usually very small (e.g. the $^{13}C/^{12}C$ ratio may vary between 0.010225 and 0.011574), it is not very practical to express these variations as differences in (ratios) of the absolute abundance of the isotopes.

Table 1.8. Average natural abundance of the main stable isotopes of carbon and nitrogen, according to Ehrlinger and Rundel, 1989

Element /isotope	Abundance (%)
Carbon	
^{12}C	98.89
^{13}C	1.11
Nitrogen	
^{14}N	99.63
^{15}N	0.37

The results are generally expressed in a standard "delta-notation" (δ) which is based on the measurement of the differences between the sample and a standard defined by the IAEA in Vienna (it is Pee Dee Belemnite for carbon and atmospheric nitrogen for nitrogen)

The stable isotope ratio (δ-value) of an element is:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13} C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Results are expressed in ‰.

1.5.2. SOURCES OF FRACTIONATION IN PRIMARY PRODUCERS

The usefullness of stable isotopes in studying element cycles and processes lies in the fact that small but significant variations observed in nature do not occur at random but are governed by fractionation processes, i.e. during a reaction (either chemical, physical or biological) the different isotope may react with different speed and this may cause the end product to have an altered isotope composition compared to the source product (reviewed by Bouillon, 2002).

1.5.2.1. Carbon-13 ($\delta^{13}C$)

The first hint of the utility of carbon isotopes for the study of trophic ecology came when the difference in the isotopic

signatures of C3 and C4 plants was discovered (reviewed by Kelly, 2000). This utility is derived from two properties: first, some sources of dietary carbon have distinct carbon-isotope signatures, and second the isotope signature of a food is incorporated into the consumer's tissues (DeNiro and Epstein, 1978).

With regards to animal ecology, the primary process that creates sources with identifiable carbon-isotope signatures is photosynthesis. Stable carbon isotopes are used most often to distinguish carbon fixed by terrestrial C3 plants from that fixed by C4 plants or marine C3 plants (table 1.9).

In aquatic plants, $\delta^{13}C$ values show a much wider range of values, which are not reliable indicator of the photosynthetic pathway used (reviewed by Bouillon et al., 2002). For instance, marine phytoplankton uses the C3 photosynthetic pathway. However, its carbon-isotope signature is significantly heavier (-22 ‰) than that of terrestrial C3 plants (-24 to –30‰). Benthic microalgae have been found to be consistently enriched in ^{13}C relative to their pelagic counterparts by an average of 7‰ (France, 1995c quoted by Bouillon, 2002). This difference between benthic and pelagic microalgae is consistent enough to be reflected in consummer $\delta^{13}C$ values and has been proposed as a valuable tool to distinguish between benthic and pelagic food sources.

Table 1.9. Comparison of $\delta^{13}C$ values in plants using different photosynthetic pathways (compiled from Descolas-gros, 1985; Lepoint et al., 2000; Bouillon, 2002). For information, the $\delta^{13}C$ of atmospheric CO_2 is –7.8‰.

	Photosynthesis carboxylating enzyme	Range of $\delta^{13}C$ values
C_4 plants	Phosphoenolpyruvate carboxylase (PEPc)	-9 to –19‰
C_3 plants	Ribulose Biphosphate Carboxylase/Oxygenase (RUBISC0)	-terrestrial plants: -24 to –30‰
		-phytoplankton: -18 to –28‰
		-Antarctic phytoplankton
		-seagrass: -15 to -3‰
		-macrophytes: -9 to –35 ‰

A multitude of factors have been found to influence the carbon isotope composition of aquatic primary producers :

- the type of substrate used (CO_2 or HCO_3^-),
- the concentration and isotopic composition of the substrate (either CO_2 or HCO_3^-),
- the growth rate,
- the cell shape and volume,
- the water flow rate and temperature.

Because phytoplankton has lighter ^{13}C values than many inshore plants (e.g., seagrasses, average $\delta^{13}C$=-10‰, range=-15 to -3‰), inshore carbon sources can sometimes be distinguished from pelagic sources (e.g., Fry, 1983, Hobson et al., 1994; Lepoint et al., 2000).
All these differences in $^{13}C/^{12}C$ ratios in primary consumers are sufficient enough to influence carbon isotopic composition ($^{13}C/^{12}C$) of their respective consumers and assign them a specific isotopic signature.

1.5.2.2. Nitrogen-15 ($\delta^{15}N$)

Unlike carbon isotopes, there is no single process, like photosynthesis, that creates a large isotopic fractionation of nitrogen isotopes in plants that can be traced through food webs. However, $\delta^{15}N$ signatures of primary producers in both aquatic and terrestrial ecosystems may be useful indicators of N sources and transformations. Fractionation may occur during various biological reaction such as:

- assimilation of dissolved inorganic nitrogen (DIN) under NO_3^-, NO_2^- or NH_4^+ form by phytoplankton or microheterotrophs (bacteria, fungi),
- nitrification and denitrification,
- N_2 fixation.

Controlled laboratory experiments have revealed wide variations with growth rate, light intensity, species, N substrate and culture conditions, resulting in fractionation values for algae between 0.7 and 23‰ (for NO_3^-), 0.7‰ (for NO_2^-), and between –9.7 and 14‰ (for NH_4^+).
However, main fractionation processes (and the most useful for our purpose) occur during trophic interactions (see below).

1.5.3. STABLE ISOTOPES AS TRACERS OF ORGANIC MATTER AND FOODWEB INTERACTIONS

The application of stable isotopes at the natural abundance level as indicators of the origin of organic matter and of trophic interactions is based upon three important hypotheses (reviewed by Bouillon et al., 2002):

1. differences (may) exist in the $\delta^{13}C$ and/or $\delta^{15}N$ signatures of different primary producers,
2. these differences are maintained or altered in a sufficiently predictable way during degradation processes,
3. consistent and predictable changes in the isotopic signature occur during transfer to higher trophic levels.

It should also be mentioned that other stable isotopes of other elements such as hydrogen, oxygen or sulphur have also been proven as useful ecological tools but are outside the scope of this introduction.

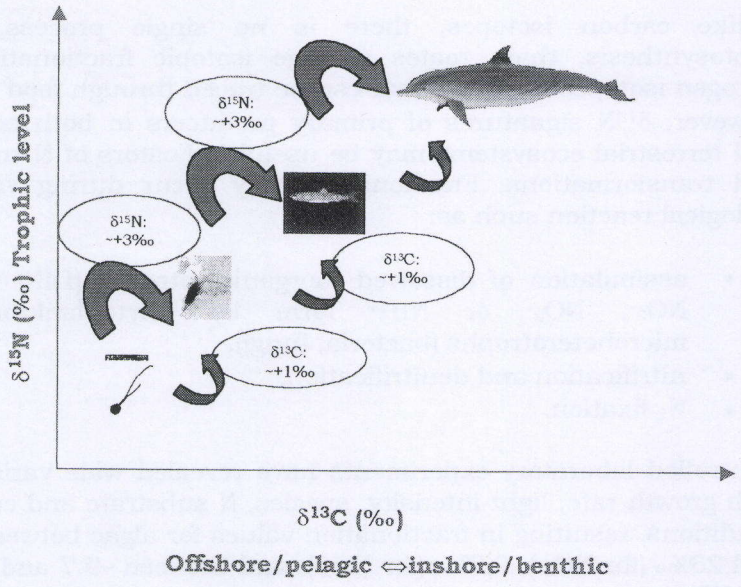

Figure 1.8. Enrichment of $\delta^{13}C$ and $\delta^{15}N$ from one trophic level to the other one

Systematic enrichment in ^{13}C values in marine food chains has been reported (Rau et al., 1983; Boutton, 1991). Most studies however, indicated that in marine environment, ^{13}C enrichment occurred at low trophic levels but not among vertebrates consumers (Rau et al., 1983; Wada et al., 1987; Fry, 1988; Hobson and Welch, 1992; Hobson, 1993; Hobson et al., 1993). This minor stepwise trophic enrichment of the carbon isotope ratio that has been documented among vertebrate consumers limits its use in assessing trophic level. However, this characteristic enhances the utility of carbon-isotope ratios for tracking carbon sources through a food chain (Peterson and Fry, 1987; Michener and Schell, 1994). Specifically, because there is little enrichment with increase in trophic level the carbon isotope signature of secondary and tertiary consumers should reflect the source of carbon (C3, C4 or marine plants) at the base of the food chain.

The variations in nitrogen isotopic composition are largely determined by biologic reactions. Catabolic pathways favour the excretion (through urine, for example) of the lighter isotope, resulting in an enrichment in animal tissues in ^{15}N relative to plants. This progressive enrichment increases along advancing trophic levels. (Ehleringer and Rundel, 1989; Minagawa and Wada, 1984 quoted by Lesage et al., 2001; Peterson and Fry, 1987).

An important advantage of this approach is that the isotope ratios from the tissues are derived from assimilated food (and not just ingested food) and therefore reflects dietary input over time (Das et al., 2000_a). This approach can also be a drawback, however, as it does not allow us to identify exactly which species has been consumed. Previous knowledge of possible prey species is therefore necessary to complement the results obtained through these analyses for trophic level studies.

The use of stable isotopes to study marine mammals is recent, but not altogether new. In the past decade we have witnessed the increase in the use of this technique for studies concerning migrations, trophic ecology, pollution and even paleontology.

Hobson and Welch (1992) characterized the trophic relationships of the food web in the Canadian arctic region of Lancaster Sound using stepwise increase in ^{15}N (and to a lesser extent ^{13}C) to

determine the trophic level of the animals concerned (zooplankton to marine mammals). This past year, (Lesage et al. 2001) published a study on marine mammals and the community structure of the Estuary and Gulf of St Lawrence in Canada, also based on stable isotope analysis. They showed that marine mammals occupy the highest trophic levels in the food web (using ^{15}N), although overlapping one another, and that an enrichment in ^{13}C occurs in animals from the Estuary relative to those from the Gulf.
Atwell et al. (1998) used $\delta^{15}N$ to characterise the trophic relationships in an arctic food web (ranging through particulate organic matter to seals and polar bears) in order to determine that food web's relationship with total Hg concentrations in the same tissues, with the objective of studying Hg bioaccumulation along the food chain and with age.

Shell et al. (2000) measured ^{13}C values in keratin in bowhead whale baleen from the western arctic environment (obtained from hunting records and museums, records extending to 1947), which can be found in growth layers. These data are representative of secondary production in the Bering Sea ecosystem and show that the ecosystem has undergone a significant decrease in average primary productivity.

In recent decades, the use of stable isotope analysis for ecological studies has been steadily increasing:

- tracer studies to determine migration patterns (reviewed by Hobson, 1999) as 'animals that move between isotopically different food webs can retain information of previous feeding locations for periods that depend on the elemental turnover rate for the tissue of interest';

- trophic ecology (Hobson and Welch, 1992; Hobson, 1999; Das et al., 2000a; Kelly, 2000; Lesage et al., 2001; Polischuk et al., 2001) to understand the relationships between organisms;

- pollution studies (Atwell et al., 1997; Thompson et al., 1998a,b; Bearhop et al., 2000, Wayland and Hobson, 2001) especially pollutant transfer through the ecosystem.

Although this dramatic increase in the application of stable isotope analyses to ecological research, this field is still in its infancy and continuous data are needed before getting a better understanding of the fundamental principles governing the

behaviour of stable isotopes in ecosystems and their potential use in solving ecological questions (Hobson and Wassenaar, 1999).

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CHAPTER 2: TUNA AND DOLPHIN ASSOCIATIONS IN THE NORTHEAST ATLANTIC: EVIDENCE OF DIFFERENT ECOLOGICAL NICHES FROM STABLE ISOTOPE AND HEAVY METAL MEASUREMENTS

After Das K., Lepoint, G., Loizeau, V., Debacker, V., Dauby, P., Bouquegneau, J.M. 2000. Marine Pollution Bulletin 40: 102-109

ABSTRACT

Associations of tunas and dolphins in the wild are quite frequent events and the question arises how predators requiring similar diet in the same habitat share their environmental resources. As isotopic composition of an animal is related to that of its preys, stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) analyses were performed in three predator species from the Northeast Atlantic: the striped dolphin, *Stenella coeruleoalba*, the common dolphin *Delphinus delphis*, and the albacore tuna, *Thunnus alalunga*, and compared to their previously described stomach content. Heavy metals (Cd, Zn, Cu and Fe) are mainly transferred through the diet and therefore have also been determined in the tissues of the animals. Tuna muscles display higher $\delta^{15}N$ than in common and striped dolphins (mean: 11.4‰ *vs.* 10.3‰ and 10.4‰, respectively) which reflects its higher trophic level nutrition. Higher $\delta^{13}C$ are found in common (-18.4‰) and striped dolphin (-18.1‰) muscles than in albacore tuna (-19.3‰) likely in relation with its migratory pattern. The most striking feature is the presence of two levels of cadmium concentrations in the livers of the tunas (32 $\mu g.g^{-1}$ *vs.* 5 $\mu g.g^{-1}$ dry weight). These two groups also differ by their iron concentrations and their $\delta^{15}N$ and $\delta^{13}C$ liver values. These results suggest that in the Biscay Bay, tunas occupy two different ecological niches likely based on different squid input in their diet.

2.1. INTRODUCTION

Small pelagic dolphins are commonly associated with tunas in mixed-species aggregations. These polyspecific associations have been extensively studied in the tropical Pacific (Perrin et al., 1973; Au and Pitman, 1986; Au, 1991; Scott and Cattanach, 1998) and comprise several species, like tunas, marine mammals, seabirds or sharks. In general, these polyspecific aggregations seem to form when social species of similar foraging habits join to form larger groups to increase feeding success and to better avoid predators (Scott and Cattanach, 1998). These animals are known to feed, interact and travel together for various periods of time (Au, 1991). In the feeding aggregations observed in tropical waters, prey is driven to the surface by yellowfin tunas and is abundant and diverse enough to allow dolphins and other species like seabirds and sharks to feed at the same time (Au and Pitman, 1986).

In the Northeast Atlantic three predator species are often caught together in the same nets during commercial seining operations: the albacore tuna (*Thunnus alalunga*), the striped dolphin (*Stenella coeruleoalba*) and the common dolphin (*Delphinus delphis*). As they are known to feed together, the question arises about the possible competition or commensal relationship that might occur between them. These kinds of alimentary studies are often performed by stomach content analysis (Perrin et al., 1973; Hassani et al., 1997) or field observations (Au and Pitman, 1986). However, these useful data reflect the most recent meal and may not be representative of the overall diet.

In marine mammals, the use of naturally occurring stable isotopes of carbon and nitrogen has provided complementary data to their feeding ecology (Hobson and Welch, 1992; Abend and Smith, 1995; Smith et al.,1996; Hobson et al., 1997; Burns et al., 1998). Stable isotope analysis has emerged as a powerful tool to trace diet as isotope ratios of a consumer are related to those of their preys (De Niro and Epstein, 1978; 1981; Peterson and Fry, 1987). Indeed, the carbon and nitrogen isotope values ($\delta^{13}C$ and $\delta^{15}N$) differ between organisms and their diets because of a slight selective retention of the heavier isotope and excretion

of the lighter one. As a result, organisms have a higher δ value than their diet. Nitrogen-15 typically shows a stepwise increase with trophic level within a food chain (Hobson and Welch, 1992; Cabana and Rasmussen, 1994; Thompson et al., 1995) with a trophic enrichment value of about 3‰ (Hobson and Welch, 1992; Michener and Schell, 1994). On the contrary, an animal has $\delta^{13}C$ values close to that of its diet (De Niro and Epstein, 1978; Hobson and Clark,1992; Thompson et al., 1995; Smith et al., 1996). Thus, carbon-13, rather than being a reliable indicator of trophic level, is generally used to indicate relative contributions to the diet of different potential primary sources in a trophic network (Rau et al., 1992; Havelange et al., 1997; Dauby et al., 1998), indicating for example the aquatic vs. terrestrial, inshore vs. offshore, or pelagic vs. benthic contribution to food intake (Hobson et al., 1995; Smith et al., 1996).

Because stable isotopes ratios of consumers' tissues are derived from assimilated food, the tissue reflects dietary input integrated over time, not just the last food intake. The metabolic rates and the subsequent turn-over of elements differ between liver and muscle, allowing the evaluation of diet integrated between different periods of time (e.g., days, week or months) (Tieszen et al., 1983; Hobson et al., 1996; 1997).

We had the opportunity to use samples from three species from the Northeast Atlantic: the striped dolphin, the common dolphin and the albacore tuna, whose stomach contents have been previously determined by Hassani et al. (1997). In order to contribute to the knowledge of ecological aspects of polyspecific associations, we have studied their isotopic composition ($\delta^{15}N$ and $\delta^{13}C$). Combined to the isotopic ratio determinations, heavy metals analysis have been performed in the tissues of the three predators. Heavy metals are merely transferred through the diet. Indeed, heavy metal levels found in marine organisms depend not only on the environmental contamination but also on several other ecological or physiological factors (Bouquegneau and Joiris, 1988; André et al., 1990a; 1990b, Caurant et al., 1994), among which the diet and the position in the trophic web are determining elements (Bouquegneau and Joiris, 1992; Cabana and Rasmussen, 1994; Kidd et al., 1995; Stewart et al., 1997).

By using a combination of stable isotopes and heavy metals analysis, our aim was to compare the diet and the position in the

trophic web of three predators from the Northeast Atlantic, the striped dolphin, the common dolphin and the albacore tuna and to contrast our results with stomach content analysis previously described by Hassani et al. (1997).

2.2. MATERIALS AND METHODS

2.2.1. COLLECTION AND STORAGE

The 23 striped dolphins (*Stenella coeruleoalba*) (10 females and 13 males) and 10 common dolphins (*Delphinus delphis*) (5 females and 5 males) used in this study originated from the Bay of Biscay in the Northeast Atlantic. The locations of the captures covered an area between 46° to 50° North and 9° to 20° West. They were accidentally captured during commercial tuna seining operations from May to September 1993, from which 20 albacore tunas (*Thunnus alalunga*) (9 females and 11 males) were collected. This collection was conducted by the *Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER)* from Brest (France) to assess ecological risk linked to the Northeast Atlantic French drif net activity (Goujon *et al.*, 1993; Goujon, 1996).

Tissue samplings were performed at the fishing site and frozen immediately in liquid nitrogen and then kept in a freezer (-20°C) until storage in the Oceanology Laboratory (Liège, Belgium).

Dolphin sex and age determinations were realised by Collet (1993a; 1993b) who showed an exponential length-age relationship (see also Di-Méglio et al., 1996). The ages of dolphins were estimated by counting tooth growth layer groups (GLGs). The total length from the snout to fluke notch were determined for each individual by Goujon (1996). All tuna specimens are juveniles (4-5 years old) with length ranging from 75 to 81 cm while the ages of the dolphins range from 0.1 to 20 years old (Collet, 1993a; b).

2.2.2. ANALYTICAL METHODS

Zn, Cd, Fe and Cu analyses

After being weighed and dried for 48 h at 110°C, samples were digested with a mixed solution of hydrochloric (Merck 317) and nitric (Merck 456) acids (1:3, v:v) and slowly heated to 100°C until complete digestion. Atomic absorption spectrophotometry (ARL 3510) was used to determine heavy metal concentrations (Cu, Zn, Cd, Fe). Pb, Ni, Cr contents were also determined but the results most often were below the detection limits (unpublished data). Concentrations are expressed as $\mu g.g^{-1}$ dry weight (dw).

Parallel to the samples, a set of certified material samples (CRM 278 Community Bureau of Reference, Commission of the European Communities) was also analysed to ensure the method's sensitivity. Recoveries ranged from 92 to 102 % for Cu, Zn, and Fe and 88% for Cd. Limits of detection were 0.01 $\mu g\ g^{-1}$ dw for Cu, 0.33 for Zn, and 0.22 for Cd.

Stable isotope measurements

Organisms may vary in their concentrations of lipids. As lipids have been shown to be depleted in ^{13}C relatively to the diet (Tieszen *et al.*, 1983), they were extracted from samples using repeated rinses with 2:1 chloroform : methanol prior to analysis. After drying at 50°C (48h), samples were ground into a homogeneous powder. Carbon dioxide and nitrogen gas were analysed on an V.G. Optima (Micromass) IR-MS coupled to a N-C-S elemental analyser (Carlo Erba). Routine measurements are precise to within 0.5‰ for 13-carbon and 0.6‰ for 15-nitrogen. Stable isotopes ratios were expressed in δ notation according to the following:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.
Carbon and nitrogen ratios are expressed relative to the VPDB (Vienna Peedee Belemnite) standard and to atmospheric nitrogen, respectively.

2.2.3. DATA TREATMENT

Parametric and non parametric tests were used to compare different groups: Kolmogorov-Smirnov test was used to assume the normality of the data. ANOVA followed by post-hoc multiple comparison tests have been used to compare the data between the different species. When the necessary assumptions to realise ANOVA were not gathered (normality of the variables and homogeneity of variances), Kruskall-Wallis was used followed by multiple comparisons based on the Kruskall-Wallis rank sums to test for pairwise differences among species. Mann-Whitney U-test was performed to compare differences among sexes or groups when variances were not homogenous. Spearman coefficient has been used to test correlations between the values. Results were judged significant when $P<0.01$ (Scherrer, 1984).

2.3. RESULTS

2.3.1. METAL LEVEL IN THE TISSUES

In dolphin and tuna tissues, Zn, Cd and Cu and Fe mean concentrations are higher in liver than in muscle (Table 2.1). In dolphins, Cd displays the highest mean concentration in kidney. Fe is always higher in the blood due to the haemoglobin respiratory pigment. In both mammal glands and testes, iron and zinc were more concentrated than copper and cadmium. Zn concentrations in the liver are similar for the three species (ANOVA, $F=1.6$, $p>0.5$). Hepatic copper levels are not significantly different for the two dolphin species but tunas display lower copper concentrations than common dolphins (ANOVA: $F=3.07$, $p<0.01$). Hepatic iron concentrations are consistently greater than those in common dolphins, which in turn are greater than those in albacore tunas (ANOVA: $F=0.94$, $p=0.04$). Cadmium concentrations in the livers are not significantly different for the three species (ANOVA: $F=0.3$, $p>0.05$). The standard deviations of cadmium mean

concentrations both in kidney and livers of dolphins (Table 2.1) are very large.

Table 2.1. Trace element concentrations (µg.g^{-1} dw), $\delta^{13}C$ and $\delta^{15}N$ (‰) of three main predators (*Stenella coeruleoalba, Delphinus delphis, Thunus alalunga*) of the Northeast Atlantic expressed as a mean ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; nd: non determined

Species		n	Zn	Cd	Cu	Fe	$\delta^{13}C$	$\delta^{15}N$
Striped dolphin *Stenella coeruleoalba*	Liver	22	167 ± 78 (33-385)	17 ± 15 (0.2-51)	43 ± 54 (7-272)	974 ± 363 (393-1792)	-17.8 ± 05 (-18.9/-17.08)	10.8 ± 0.6 (9.4-11.9)
	Muscle	23	41 ± 16 (27-101)	0.37 ± 1 (<0.05-5)	6 ± 1.4 (1-9)	577 ± 221 (96-1131)	-18.1 ± 0.5 (-19.2/-17.2)	10.4 ± 0.75 (8.8-12.1)
	Kidney	23	113 ± 24 (76-168)	91 ± 57 (0.1-199)	19 ± 6 (9-30)	716 ± 226 (413-1413)		
	Mammal gland	4	71 ± 30 (47-115)	1.3 ± 0.9 (0.4-2)	4.5 ± 1 (3-6)	273 ± 69 (187-344)	nd	
	Testis	2	45-157	0.6-3.2	3-3.2	120-215		
	Blood	12	23 ± 12 (12-52)	0.65± 0.7 (<0.05-2.5)	2.8 ± 1.3 (1.5-6)	2510± 264 (2034-2972)		
Common dolphin *Delphinus delphis*	Liver	10	153 ± 27 (117-197)	15 ± 17 (0.4-58)	32 ± 12 (19-52)	706 ± 227 (326-1096)	-17.8 ± 0.5 (-18.9/-17.3)	11.1 ± 0.4 (10.4-11.8)
	Muscle	10	50 ± 46 (27-181)	0.1 ± 0.2 (<0.05-0.5)	5 ± 1 (3-7)	470 ± 95 (278-560)	-18.4 ± 0.5 (-19.1/-17.7)	10.3 ± 0.3 (9.8-10.8)
	Kidney	10	109 ± 21 (86-146)	71 ± 61 (3-169)	20 ± 8 (27-181)	707 ± 224 (396-1032)		
	Mammal gland	4	74 ± 17 (56-97)	0.6 ± 0.9 (<0.09-2)	3.5 ± 1.4 (2-5)	188 ± 90 (110-305)	nd	
	Testis	1	99	3.1	4.2	278		
	Blood	8	21 ± 9 (13-40)	1 ± 1 (<0.07-4)	4 ± 2 (2-9)	2289 ± 504 (1153-2729)		
Albacore tuna *Thunnus alalunga*	Liver	20	152 ± 46 (107-265)	17 ± 14 (3-41)	12 ± 6 (5-29)	429 ± 343 (101-1391)	-18.4 ± 0.8 (-19.8/-16.9)	10.8 ± 0.8 (9.4-12.7)
	Muscle	12	16 ± 3 (12-21)	<0.1	1 ± 0.6 (0.5-3)	49 ± 10 (28-65)	-19.3 ± 0.6 (-20.4/-18.5)	11.4 ± 0.4 (10.9-12.2)

Metal levels are similar for both genders in the three species (data not shown), but a significant increase of this metal level with length of individuals appears in the livers of both striped and common dolphins (figure 2.1).

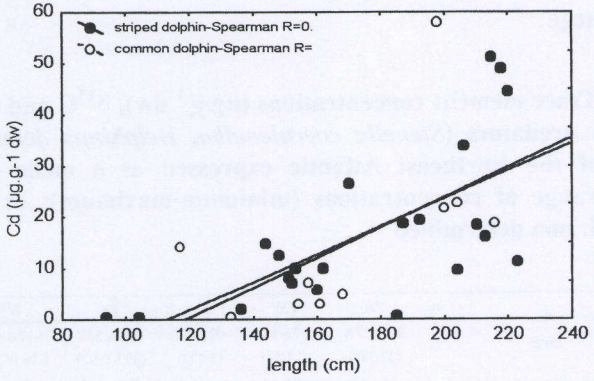

Figure 2.1. Relationship between the length of the dolphins and cadmium concentrations in livers

Such a relationship was not found between the size of the tunas and their hepatic cadmium concentrations (figure 2.2).

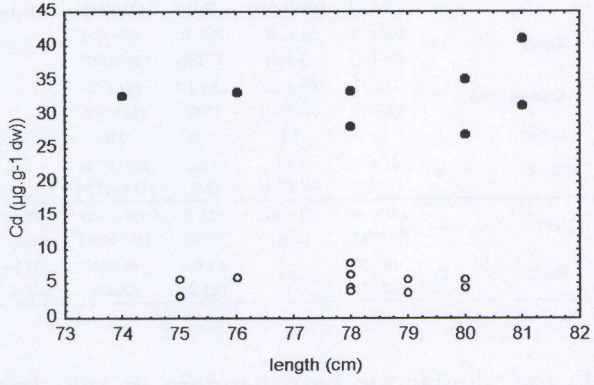

Figure 2.2. Relationship between the length of the tunas and the cadmium concentrations in the liver and. The black plots are the cadmium contaminated group.

The most striking feature of figure 2.2 is that the tuna group is heterogeneous. One group of tunas is heavily contaminated by

cadmium (mean 32 $\mu g.g^{-1}$ dw) while the other one displays lower cadmium levels (mean: 5 $\mu g.g^{-1}$ dw; Mann-Whitney U test: $p<0.001$). Zinc and copper are similar for both groups while iron, $\delta^{15}N$ and $\delta^{13}C$ are significantly different (Table 2.2). In the muscles, there are no significant differences between the two groups of tunas where cadmium concentrations were always under the detection limit.

Table 2.2. Zn, Cu, Cd, Fe ($\mu g.g^{-1}$ dw), $\delta^{15}N$ and $\delta^{13}C$ values in the livers of the two groups of tunas

	Cd contaminated group n=11	Low Cd contaminated group n=9
Zn	159 ± 4	146 ± 41
Cu	13 ± 5	10 ± 7
Cd*	32 ± 4	5 ± 1
Fe**	722 ± 312	189 ± 72
$\delta^{15}N$*	11.5 ± 0.6	10.2 ± 0.5
$\delta^{13}C$*	-18.1 ± 0.9	-18.7 ± 0.5

*significantly different with $p<0.001$ (Mann-Whitney U test)
**$p=0.05$

When the three species are compared together (Table 2.1), tunas display lower zinc and copper concentrations in muscles than the two dolphins species (Kruskall-Wallis: $p<0.001$and ANOVA: $F=1.4$, $p<0.001$, respectively).

2.3.2. STABLE ISOTOPE ANALYSES

Isotopic compositions are similar for both sexes in the three species and no effect of age has been observed ($p>0.05$, data not shown).

Tunas display significantly higher $\delta^{15}N$ in muscle than striped and common dolphins: 11.4‰ *vs.* 10.4 and 10.3‰, respectively (ANOVA: $F=3.3$, $p<0.0001$ and $p< 0.001$ respectively, Table 2.1). The mean $\delta^{15}N$ values in muscles do not vary significantly between the dolphin species: $\delta^{15}N$ of +10.3‰ in the muscles of common dolphins and +10.4‰ in the muscles of striped dolphins (ANOVA: $p>0.05$).

Tunas display a muscle $\delta^{13}C$ significantly lower than dolphins (-19.3‰ versus -18.1 and 18.4‰ for striped and common dolphins respectively (ANOVA: F=0.6, p<0.001 and p=0.001 respectively).

Individual results of $\delta^{15}N$ and $\delta^{13}C$ are plotted in figures 2.3 and 2.4. In the muscles, specific regrouping appears: tunas are completely isolated from dolphins with a higher $\delta^{15}N$ and a lower $\delta^{13}C$ (figure 2.3).

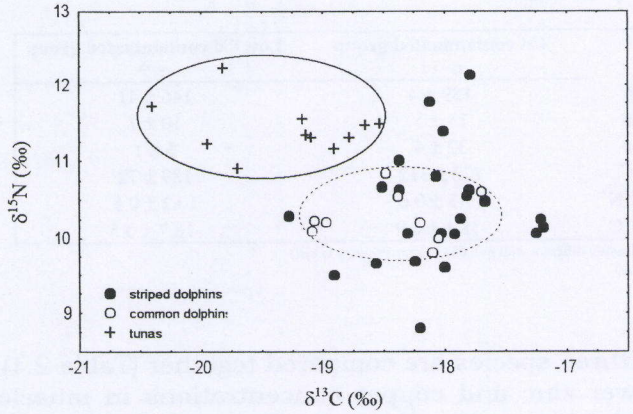

Figure 2.3. $\delta^{13}C$ and $\delta^{15}N$ in the muscles of tunas, striped and common dolphins from the Northeast Atlantic

The common dolphin data are also concentrated in a small area of the figure (medium $\delta^{15}N$ and $\delta^{13}C$ values) while the striped dolphin displays a wide range of $\delta^{15}N$ values overlapping the two other species.
In livers, no regrouping between individuals from the same species occurs (figure 2.4).

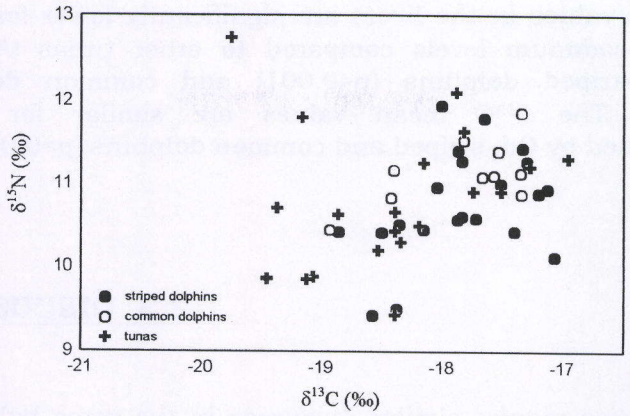

Figure 2.4. $\delta^{13}C$ and $\delta^{15}N$ in the livers of tunas, striped and common dolphins from the Northeast Atlantic

However, tunas with low cadmium contamination display lower $\delta^{15}N$ values than contaminated tunas (ANOVA: $p<0.0001$), striped and common dolphins (ANOVA: $p=0.02$ and $p<0.001$ respectively; Table 2.2, figure 2.5 and 2.6). Common dolphins display medium $\delta^{15}N$ mean values between striped dolphins and tunas with high cadmium levels, the difference between these latest being significant (ANOVA, $F= 1.17$, $p=0.002$).

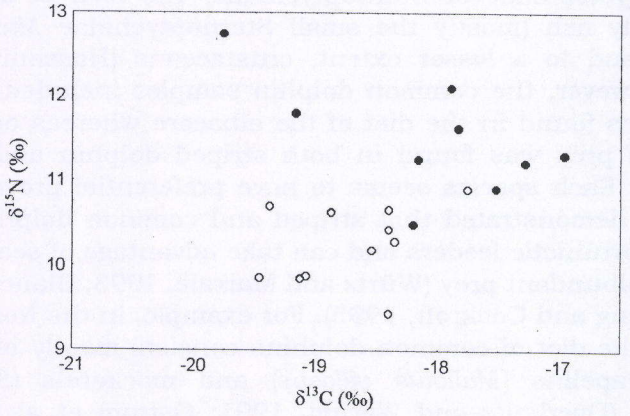

Figure 2.5. Comparison of the stable isotope values in livers between highly (•) and low-contaminated groups of tunas (o)

$\delta^{13}C$ mean values in the livers are significantly lower for tunas with low cadmium levels compared to other tunas (ANOVA: $p=0.02$), striped dolphins ($p<0.001$) and common dolphins ($p<0.002$). The $\delta^{13}C$ mean values are similar for tunas contaminated by Cd, striped and common dolphins ($p>0.05$).

2.3. DISCUSSION

When species require similar resources in the same habitat as striped and common dolphins and albacore do in the Biscay Bay, they tend to reduce the competition by occupying different physical locations or feeding on different preys, as it has already been observed for other small odontocetes (Gowans and Whitehead, 1995). A comparative dietary study of albacore and both dolphin species from Northeast Atlantic has been realised and published by Hassani et al.(1997). These authors have shown that common and striped dolphins have a more preferential cephalopod diet. Fish ranked second in the food of the common dolphin and only third in the striped dolphin, in which crustaceans were more important in relative abundance. Fish present in dolphin stomach were mostly small mesopelagic fish as Myctophidae or Sternoptychidae. The food of albacore was mainly fish (mostly the small Sternoptychidae *Maurolicus muelleri*) and to a lesser extent, crustaceans (Hassani et al., 1997). However, the common dolphin samples included all the fish species found in the diet of the albacore whereas only one species of prey was found in both striped dolphin and tunas stomachs. Each species seems to have preferential preys but it has been demonstrated that striped and common dolphins are quite opportunistic feeders and can take advantage of seasonally or locally abundant prey (Würtz and Marrale, 1993; Blanco et al., 1995; Young and Cockroft, 1995). For example, in the Northwest Atlantic, the diet of common dolphins consists mainly of bigger fish as capelins (*Mallotus villosus*) and mackerels (*Scomber scombrus*) (Overholtz and Waring, 1991; Ostrom et al., 1993). Isotopic data on delphinids are scattered (Hobson and Welch, 1992; Ostrom et al., 1993). Ostrom et al. (1993) have found in the muscles of one common dolphin from the Western North Atlantic a $\delta^{15}N$ and a $\delta^{13}C$ of 14.8 and -17.8‰, respectively. Our

isotope values in muscles of common dolphin are low compared to these data. These high $\delta^{15}N$ values measured by these authors are consistent with a higher trophic levels and the piscivorous diet of common dolphin in the studied geographical area (Ostrom et al., 1993).

Our isotopic data in muscles which integrate a long-term nutrition, also suggest a specific diet: tunas are completely isolated from the other two species with a higher $\delta^{15}N$ which reflects their higher trophic level nutrition compared with dolphins (figure 2.3). The common dolphin data are also concentrated in a small area of the figure while the striped dolphin displays a wide range of $\delta^{15}N$. This large dispersion of the individual data may reflect more opportunistic trophic habits of striped dolphins compared to common dolphin.

The trophic position of the tuna is rather uncommon because generally, in stable isotope studies, marine mammals display the highest $\delta^{15}N$ (Hobson and Welch, 1992; Ostrom et al., 1993; Hobson et al., 1995). Moreover, tunas exhibit lower $\delta^{13}C$ values than both dolphin species. Both $\delta^{15}N$ and $\delta^{13}C$ values in muscles indicate that different sources of food take part in the diet of tunas compared to striped and especially common dolphins despite a substantial overlap in diet. Tunas present a strong migratory cycle: during the spring, they are found offshore from the Azores and then migrate within the Bay of Biscay during May to August (quoted by Goujon, 1996). Measured lower $\delta^{13}C$ data presumably reflect a greater reliance on offshore food in relation with this migration. Stable carbon isotopes have proven most useful in identifying where particular organisms feed, and $\delta^{13}C$ values are typically higher in coastal or benthic food webs than in pelagic food webs (Hobson et al., 1995; 1997).

The considerable overlap in values among the three species and the absence of specific regrouping in the livers (figure 2.4) indicate isotopic similarity among their respective recent diets.

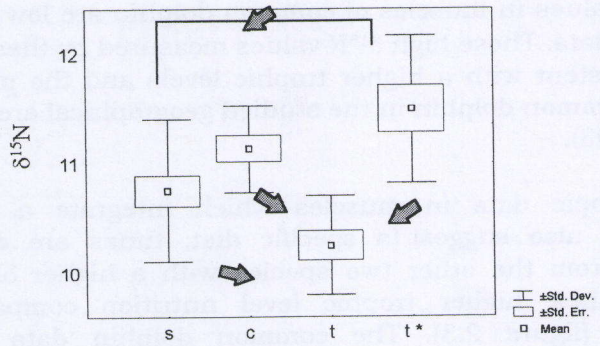

Figure 2.6. Comparison of δ15N in livers of striped dolphins (s), common dolphins (c), tunas contaminated by cadmium (t*) and other tunas (t). Arrows indicate significant decreasing difference between groups (ANOVA).

From an ecotoxicological point of view, animals feeding on cephalopods tend to concentrate higher cadmium content (Bouquegneau and Joiris, 1992; Caurant et al., 1994; Bouquegneau et al.,1997). Indeed, cephalopods have been shown to concentrate high level of this metal (Law et al., 1997). Therefore, it is quite striking to find similar cadmium levels in the livers of the three species as very few squids are found in the tuna diet (Hassani et al., 1997). However when we go through individual tuna results (figure 2.2 and table 2.2), it appears that there are two levels of iron and cadmium in the livers of tunas: one group is highly contaminated by cadmium as the other one displays low levels. There is no relationship between the size of the tunas and the hepatic cadmium concentrations. On the other hand, there is an increase of hepatic cadmium concentrations of both dolphin species with length (figure 2.1), which reflects a constant intake of cadmium contaminated preys associated with a long half-time of elimination of the pollutant (10 to 30 yr. as quoted by Wagemann et al., 1990). The absence of such a correlation in the tunas suggests that the high hepatic cadmium content might not reflect a chronic contamination but be the result of a punctual ingestion of cadmium contaminated preys. These results are enhanced by $\delta^{15}N$ and $\delta^{13}C$ differences in the livers of tunas (figure 2.5 and table 2.2). It clearly appears that isotopic composition differs strongly between the cadmium contaminated group of tunas and the second one, indicating that two different food habits were coexisting sympatrically and temporally. Differences in iron concentrations between the two groups of tunas are still to be elucidated. Cadmium levels and

isotope ratios in muscles are similar in the two groups of tunas suggesting that these feeding habits might be relatively recent.

Despite an apparent large overlap in stable isotopes values in livers of the three species (figure 2.4), different groups can be pointed out (figure 2.6). Tunas with low cadmium contamination display lower $\delta^{15}N$ and $\delta^{13}C$ mean values than others tunas and both dolphins, suggesting a greater contribution of lower trophic level preys in their diet. On the contrary, common dolphins and tunas contaminated by Cd display similar $\delta^{13}C$ and $\delta^{15}N$ values in their liver confirming that tunas share more prey similarities with common dolphins than striped dolphins (Hassani et al., 1997). In fact, common dolphins seem to occupy a medium trophic position between striped dolphins and tunas with high Cd levels (figure 2.6). These observed differences combined with cadmium concentrations in livers likely result from differential reliance on squids. Our observations are enhanced by stomach analyses carried out by Hassani et al.(1997) who have suggested that the diet of the tuna may differ: when tuna was captured alone or in the same nets as common dolphins, fish was predominant in the stomach as described earlier. However, these authors have noticed that, when tunas were captured in the same nets than striped dolphins, their stomach contents became similar to dolphins with a high squid content suggesting that feeding behaviour of albacore may differ when it co-occurs with striped dolphin. On the contrary, the presence of common dolphins would have no influence on the feeding habits of tunas (Hassani et al., 1997).

$\delta^{15}N$, $\delta^{13}C$, Fe and Cd also suggest different feeding habits between these three predators. While striped and common dolphins seem to differ little in their diet, tunas seem to occupy two potential different ecological niches in the Bay of Biscay. These different niches are based on at least two types of preys, influencing both their isotope values and their cadmium and iron content in livers. These two striking behaviours were relatively recent from the collection of the samples, in agreement with the rapid turn-over of livers compared to muscles. An experimental study carried on gerbils (*Meriones unguiculatus*) has determined a faster turn-over rate of carbon in livers (half-life: 6.4 days) than in muscles (half-life: 27.6 days) (Tieszen et al., 1983). This behaviour is likely to be in relation with the migration pattern of the tunas. When tunas get through the Biscay Gulf, some individuals become associated with dolphins

sharing similar prey preferences as squids suggesting a potential competition. For unclear reasons, some tunas display different feeding particularities mainly based on small Sternoptychidae. As tuna feeding behaviour may differ in relation with the dolphin species it is associated to, two hypotheses can be formulated considering these tunas which recently fed on Sternoptychidae :

1. Tunas and common dolphins were hunting together prey that were abundant and varied enough to allow specific preferences within a common dolphin/tuna school or, as suggested by Hassani et al. (1997)

2. These tunas were not associated with dolphins.

To conclude, combined stable isotopes and heavy metals analyses appear as powerful tool for delineating trophic relationship within these mixed-species aggregations. Further research on other organisms from this area will help to get a better understanding on the transfer of heavy metals and different alimentary relations within high trophic levels.

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CHAPTER 3: MARINE MAMMALS FROM NORTHEAST ATLANTIC : EVALUATION OF THEIR TROPHIC STATUS BY $\delta^{13}C$ AND $\delta^{15}N$ AND INFLUENCE ON THEIR TRACE METAL CONCENTRATIONS

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ABSTRACT

The relationship between trophic position through $\delta^{13}C$ and $\delta^{15}N$ and trace metal concentrations (Zn, Cd, Cu and Hg) has been investigated in the tissues of 6 marine mammal species from the Northeast Atlantic: striped dolphin *Stenella coeruleoalba,* common dolphin, *Delphinus delphis*, Atlantic white-sided dolphin *Lagenorhynchus acutus*, harbour porpoise *Phocoena phocoena*, white beaked-dolphin *Lagenorhynchus albirostris,* grey seal *Halichoerus grypus* stranded on French Channel and Irish coasts.

White-beaked dolphins, harbour porpoises, white sided dolphins, common and striped dolphins display the same relative and decreasing trophic position, as measured by $\delta^{15}N$ values, along both the Irish and French channel coasts, reflecting conservative trophic habits between these two places. Hepatic and renal Cd concentrations were significantly correlated to muscle $\delta^{13}C$ and $\delta^{15}N$ values while Hg, Zn and Cu did not. These results suggest that Cd accumulation is partly linked to the diet while other factors such as age or body condition might explain Hg, Zn or Cu variability in marine mammals. Combined stable isotope and trace metal analyses appear to be useful tools for the study of marine mammal ecology.

3.1. INTRODUCTION

Over 40 species of cetaceans and pinnipeds occur throughout the Northeast Atlantic as defined by the OSPAR Convention (OSPAR, 2000). Among these species, the common dolphin, *Delphinus delphis*, the striped dolphin, *Stenella coeruleoalba*, the harbour porpoise, *Phocoena phocoena*, the white-beaked dolphin, *Lagenorhynchus albirostris*, the white-sided dolphin, *Lagenorhynchus acutus* and the grey seal, *Halichoerus grypus* are regularly sighted within the Channel and Celtic Sea (Hammond et al., 1995; Rogan and Berrow, 1996; OSPAR, 2000).

These animals are considered to be top predators and their primordial role in the structure of the marine ecosystem has often been suggested (Bowen, 1997; Bouquegneau et al., 1997; Pauly et al., 1998). Published information on diet composition and trophic status of these species in Irish waters and the French Channel is sparse (Rogan and Berrow, 1996) and is obviously needed to understand the role of marine mammals in ecosystem dynamics. Moreover, in marine mammals, diet is the main pollutant contamination pathway and might influence their contaminant load (reviewed by Aguilar et al., 1999, Das et al., 2002).

Dietary studies are often performed by stomach content analysis or field observations. In marine mammals, the use of naturally occurring stable isotopes of carbon (^{13}C) and nitrogen (^{15}N) has recently provided new insights in the study of their feeding ecology (*e.g.* Hobson and Welch, 1992; Abend and Smith, 1995; Smith et al., 1996; Hobson et al., 1997; Burns et al., 1998; Kelly, 2000; Lesage et al., 2001). The method is based on the demonstration that stable isotope ratios of a consumer are related to those of their prey (De Niro and Epstein, 1978; 1981; Peterson and Fry, 1987). Nitrogen-15 typically shows a stepwise increase with trophic level within a food chain (Hobson and Welch, 1992; Cabana and Rasmussen, 1994; Thompson et al., 1995).

The carbon-13 value is close to that of the diet and is preferentially used to indicate relative contributions to the diet of different potential primary sources in a trophic network, indicating for example the aquatic *vs.* terrestrial, inshore *vs.* offshore, or pelagic *vs.* benthic contribution to the food intake (Hobson et al., 1995; Smith et al., 1996; Dauby et al., 1998).

When using stable isotopes to assess diets of animals feeding at or near the top of the trophic web on several and on different prey items, many of which may have similar isotopic signatures, clear distinctions about the diet are more difficult to determine. However, it may be possible to infer the general trophic level at which animals are feeding, by applying and comparing ^{15}N (and to a limited extent ^{13}C) step-wise enrichment values (Kurle and Worthy, 2001). Furthermore, stable isotope analysis is often used to provide a continuous variable with which to assess both trophic level (Michener and Schell, 1994; Hobson et al., 1995) and trophic transfer of contaminants (Kidd et al., 1995; Das et al., 2000).

In previous studies, stable isotope ratios and trace metal concentrations were determined in common and striped dolphin tissues from the Northeast Atlantic (chapter 2) and high renal Cd concentrations encountered were assumed to be related to the diet.

In this study, we examine closer and compare the relationship among 6 marine mammal species from the Irish and French channel coasts, the grey seal, the harbour porpoise, the striped dolphin, the common dolphin, the white-sided dolphin and the white-beaked dolphin, using a multidisciplinary approach based on stable isotope ($\delta^{13}C$ and $\delta^{15}N$) and trace metal analyses. We also examined mercury (Hg), cadmium (Cd), zinc (Zn) and copper (Cu) concentrations for evidence of diet transfer, specific bioaccumulation or biomagnification processes and differences between the two regions.

3.2. MATERIALS AND METHODS

3.2.1. COLLECTION AND STORAGE

Liver, kidney and muscle samples were collected from 7 striped dolphins, 24 common dolphins, 13 harbour porpoises, 4 white-beaked dolphins, 5 white-sided dolphins and two grey seals found stranded (while some of them are be caught suspected) in fishing nets along the Irish coast between 1989 and 1993 (counties of Cork, Galway, Kerry, Meath, Clare and Waterford) and the northern French Atlantic coast (region of Cotentin) between 1998 and 2001.

3.2.2. ANALYTICAL METHODS

Zn, Cd and Cu analyses

After being weighed and dried for 48 h at 110°C, samples were digested with a solution of nitric acid (Merck 456) and slowly heated to 100C° until complete digestion. Atomic absorption spectrophotometry (ARL 3510) was used to determine Cu, Zn and Cd concentrations. Concentrations are expressed as $\mu g\ g^{-1}$ dry weight (dw).
Parallel to the samples, a set of certified material samples (CRM 278 Community Bureau of Reference, Commission of the European Communities) was also analysed to ensure the method's sensitivity. Recoveries ranged from 92 to 102 % for Cu and Zn, and 88% for Cd. Limits of detection were 0.01 $\mu g\ g^{-1}$ dw for Cu, 0.33 for Zn, and 0.22 for Cd.

Hg analyses

Hg was analysed by flameless atomic absorption spectrophotometry (Perkin-Elmer MAS-50A) after sulfuric acid

digestion, as described by Joiris et al., 1991). Quality control measurements for total mercury included replicate analysis resulting in coefficients of variation <10% and analysis of certified material (DORM-1, NRC, Canada).

Stable isotope measurements

Organisms may vary in their concentrations of lipids. As lipids have been shown to be depleted in ^{13}C relatively to the diet (Tieszen et al., 1983), they were extracted from samples using repeated rinses with 2:1 chloroform: methanol prior to analysis. After drying at 50°C (48h), samples were ground with a mortar and pestle into powder. Stable isotope measurements were performed on a V.G. Optima (Micromass) IR-MS coupled to a N-C-S elemental analyser (Carlo Erba) for automated analyses. Routine measurements are precise to within 0.3 $^{0}/_{00}$ for 13-carbon and 15-nitrogen. Stable isotope ratios were expressed in δ notation according to the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.
Carbon and nitrogen ratios are expressed relative to the V-PDB (Vienna Peedee Belemnite) standard and to atmospheric nitrogen, respectively. Reference materials were IAEA-N1 ($\delta^{15}N$ = +0.4 ± 0.2‰) and IAEA CH-6 (sucrose) ($\delta^{13}C$ = -10.4 ± 0.2‰).

3.2.3. DATA TREATMENT

Kolmogorov-Smirnov test was used to test for data departure to normality. When not distributed normally the variables were log-transformed to normalize their distribution. Effect of species and sampling location on $\delta^{13}C$ and $\delta^{15}N$ values or trace metal concentrations were tested simultaneously using multivariate analysis of variance (2-way MANOVA) followed by post-hoc multiple comparison tests (LSD test). Parametric Spearman-coefficient has been used to test correlations between the values. Results were judged significant when $p<0.01$ unless otherwise stated.

3.3. RESULTS

3.3.1. STABLE ISOTOPE ANALYSIS

$\delta^{15}N$ and $\delta^{13}C$ analyses were performed in the muscles of six marine mammal species (table 3.1). The grey seal and the white-sided dolphin were excluded from statistical treatment due to the small sample size and unavaibility for the two regions.

Table 3.1. $\delta^{15}N$ and $\delta^{13}C$ values in muscles of marine mammals from the French Channel and Irish coasts. Data is given as average (median) ± standard deviation, (minimum – maximum); n: number of samples, na: not available.

	Channel coast		Irish coast	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Striped dolphin *Stenella coeruleoalba*	-16.7 ± 0.4 (-17.1 / -16.4) n=3	11 ± 1.8 (9.8 - 13.1) n=3	-17.5 ± 0.1 (-17.7 / -17.4) n=3	10.8 ± 0.6 (10.2 - 11.3) n=3
Common dolphin *Delphinus delphis*	-16.5 ± 0.5 (-17.1 / -15.6) n=8	12.1 ± 0.4 (11.4 - 12.6) n=8	-17.1 ± 0.4 (-18 / -16.5) n=14	12.2 ± 1 (10 - 14.3) n=14
Atlantic white-sided dolphin *Lagenorhynchus acutus*	na		-17.0 ± 0.5 (-17.4 / -16.4) n=4	12.7 ± 0.5 (12.1 - 13.4) n=4
Harbour porpoise *Phocoena phocoena*	-16.1 ± 0.6 (-17.1 / -15.8) n=4	16.5 ± 2.4 (13.2 - 18.8) n=4	-16.5 ± 0.7 (-17.2 / -15.1) n=7	14.1 ± 1.6 (12.0 - 17.2) n=7
White-beaked dolphin *Lagenorhynchus albirostris*	-15.4 n=1	16.5 n=1	-16.3 ± 0.3 (-16.6 / -16.0) n=3	15.8 ± 2.3 (13.3 - 17.8) n=3
Grey seal *Halichoerus grypus*	-15.4 n=1	18.3 n=1	na	

$\delta^{13}C$ measurements varied significantly with both species and sampling location while mean $\delta^{15}N$ value remained similar between French Channel and Irish coasts (2-way MANOVA, univariate results see table 3.4). In both ecosystems, the lower $\delta^{15}N$ values were displayed by the striped dolphin, significantly depleted compared to harbour porpoise (post-hoc LDS test, $p<0.0001$) and white-beaked dolphin (post-hoc LSD test, $p<0001$). Mean $\delta^{15}N$ data did not differ significantly between striped and common dolphins (post-hoc LSD test, $p=0.06$). For both ecosystems, striped dolphins were significantly depleted in carbon-13 compared to white-beaked dolphin (post-hoc LSD test, $p<0.0005$) and harbour porpoises (post-hoc LSD test, $p<0.005$) but were similar to common dolphins (post-hoc LSD test, $p=0.1$). Mean $\delta^{13}C$ values did not differ significantly between harbour porpoise and white-beaked dolphin ($p>0.1$).
The mean muscle and liver $\delta^{13}C$ values were significantly more negative for the animals from the Irish coasts compared to animals from the Northern French coast (post-hoc LSD test, $p<0.0002$)

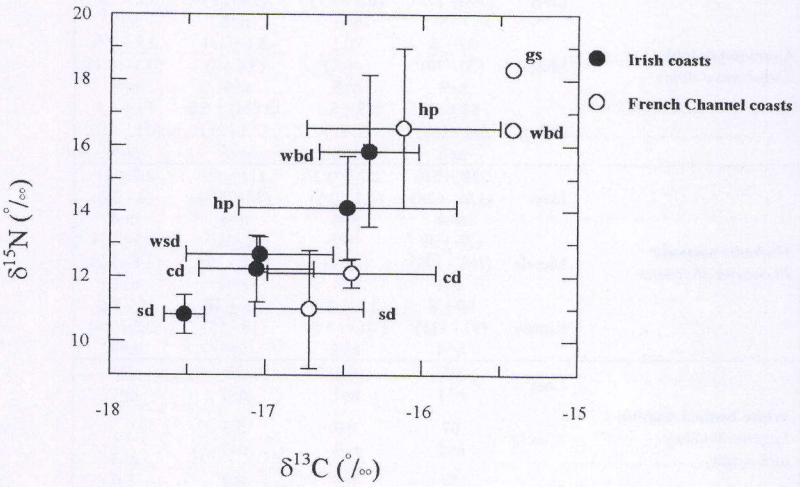

Figure 3.1. Muscle $\delta^{13}C$ and $\delta^{15}N$ in marine mammals from Irish, French Channel coasts (sd: striped dolphin; cd: common dolphin; wsd: white-sided dolphin; hp: harbour porpoise; wbd: white-beaked dolphin, gs: grey seal

3.3.2. METAL LEVEL IN THE TISSUES

Geographic location did not affect trace metal concentrations while Zn, Cu and Cd displayed strong-interspecific differences (table 3.2 and 3.3). Hg remained similar between species and locations.

Table 3.2. Zn, Cd, Cu and Hg concentrations ($\mu g.g^{-1}$ dry weight) in the liver, muscle and kidney of marine mammals from the French Channel coasts, data is given as a mean (median) ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; na: not available

		Zn	Cd	Cu	Hg
Striped dolphin *Stenella coeruleoalba*	Liver	140 ± 41 (94 - 171) n=3	35 ± 55 (3 - 99) n=3	26 ± 9.2 (18 - 36) n=3	37 ± 25 (11 - 60) n=3
	Muscle	33 ± 0.9 (33 - 34) n=3	<0.1 ± 0.2 (<0.1 / 0.3) n=3	7.8 ± 1.2 (6.9 - 9.1) n=3	3.7 ± 1.5 (2 - 4.8) n=3
	Kidney	112 ± 35 (73 - 140) n=3	71 ± 104 (<0.1 / 190) n=3	16 ± 2.6 (14 - 19) n=3	8 ± 4.3 (3.9 – 13) n=3
Common dolphin *Delphinus delphis*	Liver	141 ± 31 (106 - 177) n=5	1.5 ± 1.3 (0.3 - 3.7) n=5	17 ± 3 (14 - 22) n=5	124 ± 156 (2.2 - 320) n=5
	Muscle	32 ± 6 (20 - 39) n=8	<0.1 (<0.1) n=8	5.1 ± 1.4 (3.8 - 7) n=8	2.7 ± 2.9 (0.3 – 9.1) n=8
	Kidney	84 ± 23 (55 - 108) n=5	8.7 ± 8.1 (1-22) n=5	13 (14) ± 5.6 (7.5 - 21) n=5	13 ± 5.1 (6.8 – 20) n=5
Harbour porpoise *Phocoena phocoena*	Liver	258 ± 246 (126 - 628) n=4	0.3 ± 0.2 (0.1 - 0.5) n=4	111 ± 69 (36 - 203) n=4	8.9 ± 11 (3 - 26) n=4
	Muscle	126 ± 49 (101 - 199) n=4	<0.1 (<0.1) n=4	12 ± 4.6 (5.2 - 15) n=4	3.5 ± 2.4 (1.8– 5.2) n=2
	Kidney	99 ± 8 (93 - 111) n=4	1.5 ± 2.7 (<0.1 / 5.6) n=4	32 ± 16 (18 - 55) n=4	4 ± 2.5 (2.3 - 7.6) n=4
White-beaked dolphin *Lagenorhynchus albirostris*	Liver	96 n=1	0.4 n=1	27 n=1	229 n=1
	Muscle	67 n=1	0.9 n=1	5.5 n=1	4.2 n=1
	Kidney	37 n=1	0.9 n=1	8.2 n=1	5.6 n=1
Grey seal *Halichoerus grypus*	Liver	204 ± 59 (163 - 246) n=2	1.5 ± 1.0 (0.8 - 2.2) n=2	51 ± 7 (46 - 56) n=2	368 ± 59 (326 - 409) n=2
	Muscle	92 n=1	0.3 n=1	3.7 n=1	7.6 n=1
	Kidney	150 n=1	5.7 n=1	17 n=1	49 n=1

Table 3.3. Zn, Cd, Cu and Hg concentrations ($\mu g.g^{-1}$ dry weight) in the liver, muscle and kidney of marine mammals from the Irish coasts Data is given as a mean (median) ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; na: not available

		Zn	Cd	Cu	Hg
Striped dolphin *Stenella coeruleoalba*	Liver	185 ± 60 (133 - 250) n=3	38 ± 13 (25 - 51) n=3	39 ± 9 (30 - 49) n=3	41 ± 31 (19 - 63) n=2
	Muscle	47 ± 9 (42 - 58) n=3	0.2 ± 0.2 (<0.1 - 0.4) n=3	5.9 ± 1.7 (4.7 - 7.8) n=3	4 ± 0.9 (3.4 - 4.6) n=2
	Kidney	150 ± 15 (136 - 163) n=4	150 ± 35 (118 - 199) n=4	18 ± 4.6 (13 - 24) n=4	15 ± 5 (11 - 18) n=2
Common dolphin *Delphinus delphis*	Liver	150 ± 35 (81 - 220) n=14	6.8 ± 6.9 (1.2 - 27) n=14	20 ± 4.6 (13 - 28) n=14	46 ± 55 (4 -1 63) n=8
	Muscle	48 ± 13 (35 - 80) n=14	0.3 ± 0.3 (<0.1-1) n=14	6 ± 1.7 (3.6 - 9.8) n=14	2.7 ± 1.2 (1.5 – 4.9) n=3
	Kidney	97 ± 23 (53 - 130) n=12	33 ± 25 (6 - 72) n=12	13 ± 3.2 (8.2 – 19) n=12	8 ± 5 (2 - 14) n=7
White-sided dolphin *Lagenorhynchus acutus*	Liver	136 ± 140 (37 - 235) n=2	2.8 ± 4 (<0.1 / 5.7) n=2	18 ± 22 (2.7 - 33) n=2	150 n=1
	Muscle	41 ± 24 (22 - 75) n=4	3.6 ± 6.7 (0.1 - 14) n=4	4.8 ± 1.7 (3.2 - 6.4) n=4	2.7 ± 1.2 (1.5 – 3.9) n=3
	Kidney	97 ± 10 (90 - 104) n=2	9.6 ± 14 (<0.1 / 19) n=2	7.7 ± 6.2 (3.4 - 12) n=2	7.8 n=1
Harbour porpoise *Phocoena phocoena*	Liver	173 ± 98 (91 - 380) n=8	0.6 ± 0.4 (<0.1 / 1.1) n=8	22 ± 11 (3 - 39) n=8	24 ± 42 (4.1 – 99) n=5
	Muscle	45 ± 8 (32 - 56) n=7	<0.1 ± 0.1 (<0.1 / 0.2) n=7	4.6 ± 1.6 (1.2 - 6) n=7	3.6 ± 1.4 (2.5 – 5.6) n=4
	Kidney	90 ± 16 (70 - 112) n=7	3.9 ± 3.4 (0.4 - 10) n=7	13 ± 1.6 (11 - 15) n=7	2.2 ± 0.6 (1.6 – 2.9) n=4

Table 3.3. (continued). Zn, Cd, Cu and Hg concentrations (µg.g^{-1} dry weight) in the liver, muscle and kidney of marine mammals from the Irish coasts Data is given as a mean (median) ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; na: not available

		Zn	Cd	Cu	Hg
White-beaked dolphin *Lagenorhynchus albirostris*	Liver	101 ± 28 (81 - 121) n=2	0.3 ± 0.1 (0.2 - 0.3) n=2	24 ± 14 (14 - 34) n=2	na
	Muscle	97 ± 39 (53 - 127) n=3	<0.1 (<0.1) n=3	5 ± 2 (3.5 - 7.2) n=3	
	Kidney	73 ± 41 (44 - 102) n=2	0.3 ± 0.03 (0.2 - 0.3) n=2	7.6 ± 6.1 (3.3 - 12) n=2	

Striped dolphin displayed higher renal Zn concentrations than harbour porpoise (post-hoc LSD test, $p<0.01$) and common dolphins (post-hoc LSD test, $p<0.005$), which in turn were higher than those of white-beaked dolphin (post-hoc LSD test, $p<0.05$).

Striped dolphin displayed the highest hepatic and renal Cd concentrations significantly higher than those of common dolphins (post-hoc LSD test, $p<0.0006$), which in turn were higher than those of harbour porpoise (post-hoc LSD test, $p<0.0001$) and white beaked dolphin (post-hoc LSD test, $p<0.01$). The highest hepatic Cu was measured in the liver of the harbour porpoise and the lowest in the common dolphin (post-hoc LSD test, $p<0.005$). Other species displayed similar Cu concentrations.

Table 3.4. Influence of species and sampling locations (2-way MANOVA results, univariate specific effects) on metal concentrations and stable isotope ratios in the tissues. ns not significant, p>0.1; log: indicate the data were log-transformed before statistical treatment to ensure a normal distribution

Factors	species	geographic location
	Liver	
Zn (log)	$F_{3,23}$=1.4, p>0.2	$F_{1,23}$=0.001, p>0.9
Cu (log)	**$F_{3,23}$=4.1, p<0.02**	$F_{1,23}$=0.9, p>0.3
Cd (log)	**$F_{3,26}$=20.2, p<0.0001**	$F_{1,26}$=0.6, p>0.4
Hg (log)	$F_{3,14}$=1.2, p>0.3	$F_{1,14}$=0.7, p>0.4
	Kidney	
Zn (log)	**$F_{3,23}$=5.9, p<0.005**	$F_{1,23}$=0.16, p>0.6
Cu (log)	**$F_{3,23}$=4.7, p<0.015**	$F_{1,23}$=3.9, p>0.06
Cd (log)	**$F_{3,26}$=20.3, p<0.0001**	$F_{1,26}$=1.15, p>0.2
Hg	$F_{3,14}$=0.8, p>0.5	$F_{1,14}$=0.05, p>0.8
	Muscle	
Zn (log)	**$F_{3,23}$=12.2, p<0.0001**	$F_{1,23}$=2.98, p>0.09
Cu (log)	$F_{3,23}$=0.6, p>0.6	$F_{1,23}$=3.3, p>0.08
Hg (log)	$F_{3,14}$=0.06, p>0.9	$F_{1,14}$=0.001, p>0.9
$\delta^{13}C$	**$F_{3,35}$=6.4, p<0.002**	**$F_{1,35}$=11.7, p<0.002**
$\delta^{15}N$	**$F_{3,35}$=22.01, p<0.0001**	$F_{1,35}$=2, p>0.1

3.3.3. RELATIONSHIP BETWEEN STABLE ISOTOPES AND HEAVY METALS

No clear relationship was observed between hepatic or muscular $\delta^{13}C$ and $\delta^{15}N$ and hepatic, renal or muscular Hg concentrations, neither within a species nor for all species considered (p>0.01).

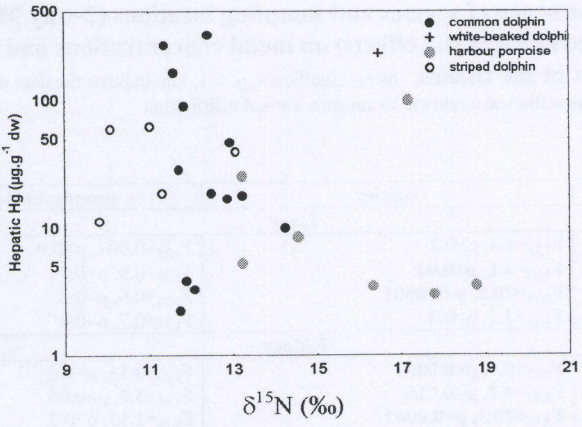

Figure 3.2. Relationship between muscle $\delta^{15}N$ and hepatic Hg concentration using a log-scale in marine mammals from Irish and French Channel coasts

A decreasing relationship was observed between muscle $\delta^{15}N$ and cadmium concentrations (log-transformed) in the liver (Pearson Product-Moment Correlation, r=-0.68, p<0.0001, n=35) and in the kidney (Pearson Product-Moment Correlation, r=-0.76, p<0.0001, n=31, figure 3.3).

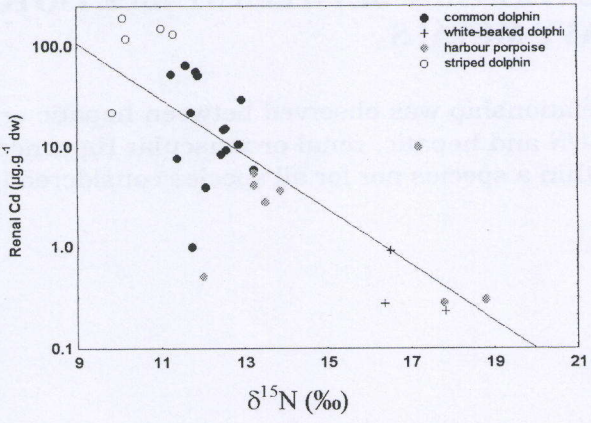

Figure 3.3. Relationship between muscle $\delta^{15}N$ and renal Cd concentration using a log-scale in marine mammals from Irish and French Channel coasts

A similar correlation was found between $\delta^{13}C$ values and cadmium concentrations (log-transformed) in the liver (Pearson Product-Moment Correlation, r=-0.47, p<0.005, n=35) and in the kidney (Pearson Product-Moment Correlation, r=-0.56, p<0.001, n=31, figure 3.4).

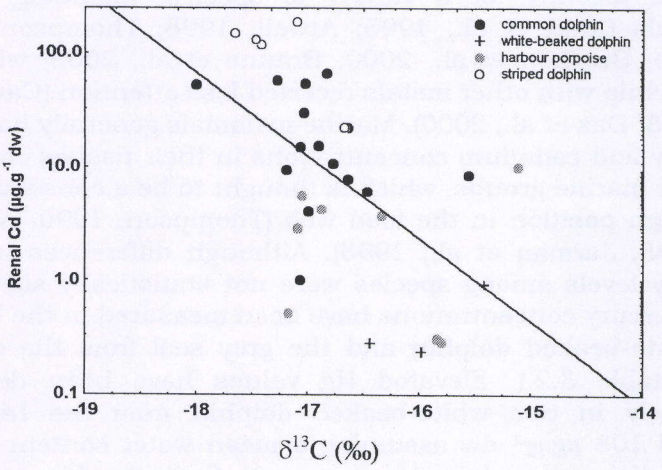

Figure 3.4. Relationship between muscle $\delta^{13}C$ and renal Cd concentration using a log-scale in marine mammals from Irish and French Channel coasts

Zn and Cu in liver, kidney or muscle were never correlated with either muscle $\delta^{13}C$ and $\delta^{15}N$ values.

3.4. DISCUSSION

The usefulness of $\delta^{13}C$ and $\delta^{15}N$ signatures as a measure of trophic status in studies of mercury accumulation has been reported recently for a variety of species, including marine mammals (Kidd et al., 1995; Atwell, 1998; Thompson et al., 1998a,b; Bearhop et al., 2000; Braune et al., 2002) while the relationship with other metals received less attention (Camuso et al., 1998; Das et al., 2000). Marine mammals generally have high mercury and cadmium concentrations in their tissues compared to other marine groups, which is thought to be a consequence of their high position in the food web (Thompson, 1990, Atwell et al., 1998; Jarman et al., 1998). Although differences in mean mercury levels among species were not statistically significant, high mercury concentrations have been measured in the livers of the white-beaked dolphin and the grey seal from the channel coast (table 3.2.). Elevated Hg values have been described previously in one white-beaked dolphin from the Irish Sea (around 108 $\mu g.g^{-1}$ dw assuming a mean water content of 75% for the tissues) and in 12 grey seals from the Liverpool Bay (mean: 590 $\mu g.g^{-1}$ dw) (Law et al., 1992). Positive correlations between $\delta^{15}N$ values and Hg concentrations have in the past been described for freshwater fish species (Kidd et al., 1995), in the blood of the great skua chicks (Bearhop, et al., 2000) or more generally within a trophic web (Atwell et al., 1998), suggesting that part of the Hg variation can be linked to a bioamplification process.

In the present study we did not find any positive relationship between $\delta^{15}N$ (or $\delta^{13}C$) values and mercury concentrations either for all species together (figure 3.2), or within a single species while the wide range of $\delta^{13}C$ and $\delta^{15}N$ values observed among the six marine mammal species supports the hypothesis of a different trophic status (figure 3.1). Indeed, when considering all the individuals together, $\delta^{15}N$ values range from 9‰ to more than 19‰.

The $\delta^{13}C$ and $\delta^{15}N$ value in marine predator tissues is determined initially by the isotopic composition of the baseline phyto- and zooplankton sources, technically measured in the particulate

organic matter (POM). No POM data are available for the Celtic Sea or the French Channel. A mean $\delta^{15}N$ value of 5‰ is generally used for offshore POM (Tucker et al., 1999) but data may vary with their sampling origin, from 4.5 ‰ in the Gulf of St-Lawrence (Northwest Atlantic) to 5.0±1.2 ‰ in some salt marshes within the Northeast French Atlantic (Lesage et al., 2001; Riera et al., 1999), reaching even higher values (up to 9‰) in the coastal part of the North Sea (Mariotti et al., 1994; Middelburg and Nieuwenhuize, 1998). As a result, part of the $\delta^{15}N$ interspecific variation in marine mammals might be related to coastal *versus* offshore $\delta^{15}N$ signature of the primary producers. Indeed, some species such as the striped dolphin are typically oceanic while others, such as the harbour porpoise, are a more coastal (Evans et al., 1987). However, these geographic differences observed for particulate organic matter (POM) do not explain the large $\delta^{15}N$ range displayed by the 6 marine mammal species of this study and these $\delta^{15}N$ values reflect specific trophic status. In both areas, high muscle and hepatic $\delta^{15}N$ values encountered suggest a higher trophic position of white beaked dolphins and harbour porpoises compared to common or striped dolphins. A high $\delta^{15}N$ value has also been measured in the grey seals but must be confirmed on a larger sampling (figure 3.1). Moreover, white-beaked dolphins, harbour porpoises, white sided dolphins, common and striped dolphins display the same relative and decreasing trophic position, as measured by $\delta^{15}N$ values, along both the Irish and French Channel coasts, reflecting conservative trophic habits between these two places.

While previous studies suggest that mercury levels tend to be greater in tissues of higher trophic level organisms, it is unclear to what extent this is the result of biomagnification through the food web or bioaccumulation within organisms over time (Atwell et al., 1998). This is difficult to discern as top-predators such as marine mammals are long-living species and results are weakened by the absence of age data. Relationships between Hg and age have been reported for various porpoise (Siebert et al., 1999), dolphin (Honda and Tatsukawa, 1981; Honda et al., 1983) or seal species (Anan et al., 2002). In this study, total concentrations of mercury were measured, but it is well known that organic and inorganic species of mercury have very different dynamics in marine mammals. Methyl-mercury is the main form present in the prey (fish and invertebrates) and then is stored

indefinitely as tiemmanite (HgSe) in the liver of marine mammals (Nigro and Leonzio, 1996).

In contrast to Hg, a significant decreasing relationship between muscular $\delta^{13}C$ and $\delta^{15}N$ values and renal Cd suggest that some of the variation can be linked to dietary specialisation (figures 3.3 and 3.4). The tendency observed is, as muscle $\delta^{15}N$ and $\delta^{13}C$ increase, cadmium concentrations decrease, with values ranging between those of striped dolphins (maximum renal cadmium, minimum muscle $\delta^{15}N$ and $\delta^{13}C$) and grey seals and white-beaked dolphins (minimum renal cadmium, maximum muscle $\delta^{15}N$ and $\delta^{13}C$).

$\delta^{13}C$ is more useful to indicate the origin of carbon sources than as an indicator of the trophic level. The general pattern of inshore, benthos linked food webs being more enriched in ^{13}C compared with offshore, pelagic food webs presents a potentially useful tool. For example, $\delta^{13}C$ values are typically higher in coastal or benthic food webs than in offshore food webs (Hobson, 1999). A clear relation is observed between increasing muscle $\delta^{13}C$ of the six marine mammal species and oceanic *versus* coastal habitat preference (figure 3.1). The $\delta^{13}C$ depletion observed for striped, common and white-sided dolphins would therefore presumably reflect a greater reliance on offshore food while the higher mean values observed for harbour porpoise, white-beaked dolphin and grey seal correspond to their preference for a more coastal habitat (figure 3.1). The white-beaked dolphin has less oceanic preferences than the Atlantic white-sided dolphin (Cipriano, 2002; Kinze, 2002) which is in agreement with its higher $\delta^{13}C$ values. High Cd concentrations have been reported previously in the livers and kidneys of by-caught striped and common dolphins from the Northeast Atlantic (Das et al., 2000) and oceanic cephalopods constituted a significant part of their diet (Hassani et al., 1997). Previous studies have highlighted the ability of cephalopods to concentrate cadmium in the digestive gland, even in unpolluted areas like the Kerguelen Islands (Bustamante, et al., 1998b). Oceanic cephalopods are, indeed, considered an essential link for cadmium transfer in marine trophic food chains (Law et al., 1997; Bustamante et al., 1998a). Our results suggest that the high Cd values encountered in striped and common dolphins from the Irish and the French Channel coasts are partly diet related as a result of ingestion of prey displaying low $\delta^{15}N$ and

$\delta^{13}C$ values and high Cd levels. Such high Cd levels can be found in oceanic cephalopods (Bustamante et al., 1998) which furthermore display typical low $\delta^{15}N$ values (Ostrom et al., 1993, Hooker et al., 2001) compared to individuals collected within the Southern North Sea bight (see chapter 4). In contrast harbour porpoises, white-beaked dolphins and grey seals are likely to rely more on fish species. A previous stomach content study reported that fish species such as Gadidae and Clupeidae form the bulk of harbour porpoise diet (Rogan and Berrow, 1996).

No relation was found between isotopic composition and either Zn or Cu in the tissues suggesting that the large value range observed for these species is not diet related. Other factors such as body condition influenced by nutritional stress are likely to be involved. Zn, Cu and Cd values measured in these stranded common and striped dolphins are similar to that reported for the same species individuals by-caught in the Bay of Biscay in 1993 (Das et al., 2000). Harbour porpoises that died from infectious diseases displayed significantly higher Zn and Hg concentrations than healthy porpoises that died from physical trauma while Cu and Cd did not differ between the two groups (Bennet et al., 2001). Previous studies have also documented an increase of $\delta^{15}N$ values in starving animals as they might use their proteins for survival (Gannes et al., 1998). In birds, nutritional stress caused substantial increases in diet-fractionation values due either to mobilization and redeposition of proteins elsewhere in the body or amino acid composition changes in the tissues (Hobson and Clark, 1992; Gannes et al., 1998). In contrast, Arctic ground squirrels (*Spermophilus parryii plesius*) in poor and excellent body condition had similar $\delta^{15}N$ values (Ben-David et al., 1999). Similarly, muscle $\delta^{15}N$ and $\delta^{13}C$ values do not differ between porpoises from the North Sea displaying a poor, moderate of good body condition allowing the use of their muscle tissue for stable isotope studies (see chapter 5).

To conclude, moreover, white-beaked dolphins, harbour porpoises, white sided dolphins, common and striped dolphins display the same relative and decreasing trophic position, as measured by $\delta^{15}N$ values, both the Irish and French channel coasts, reflecting conservative trophic habits between these two places.
Hepatic and renal Cd concentrations were significantly correlated to muscle $\delta^{13}C$ and $\delta^{15}N$ values while Hg, Zn and Cu did not. These results suggest that Cd accumulation is partly

linked to the diet while other factors such as age or body condition might explain Hg, Zn or Cu variability in marine mammals. Combined stable isotope and trace metal analyses appear as promising and powerful tools for the study of marine mammal ecology. Further work should concentrate on using the stable isotope method to further explore the behaviour and transfer of trace metals in the marine environment. However, further data on other trophic components from the Northeast Atlantic should allow a better understanding of trophic contaminant transfer.

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CHAPTER 4: MARINE MAMMALS FROM THE SOUTHERN NORTH SEA: INSIGHTS IN THEIR FEEDING ECOLOGY FROM $\delta^{15}N$ AND $\delta^{13}C$ MEASUREMENTS

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ABSTRACT

The harbour porpoise *Phocoena phocoena*, the harbour seal *Phoca vitulina*, the grey seal *Halichoerus grypus* and the white-beaked dolphin *Lagenorhynchus albirostris* are regularly found stranded along the Southern North Sea coast. More occasionally, offshore species such as the white-sided dolphin *Lagenorhynchus acutus*, the sperm whale *Physeter macrocephalus* or the fin whale *Balaenoptera physalus* are also found stranded. In order to trace their diet, $\delta^{15}N$ and $\delta^{13}C$ measurements have been performed in their organs as well as in 49 invertebrate and fish species collected in the Southern North Sea. $\delta^{15}N$ data suggest that harbour seal, grey seal and white-beaked dolphin occupy the highest trophic position along with ichtyophageous fish such as gadids (mean muscle values: 18.7‰, 17.9‰, 18.8‰ and 17.9‰ respectively). Harbour porpoise occupies a slightly lower trophic position (mean $\delta^{15}N$ value: 16.2‰) reflecting a higher amount of zooplanktivorous fish in their diet (mean $\delta^{15}N$: 14.7 ‰). Suckling harbour porpoises display a significant $\delta^{15}N$ enrichment of 2.8‰ compared to adult females. Adult females are ^{15}N-enriched compared to adult male harbour porpoises. Sperm whales, fin whales and white-sided dolphins are ^{13}C-depleted compared to Southern North Sea POM and species suggesting that despite regular sightings, they basically (if at all) do not feed within the Southern North Sea area. Fish relying on plankton such as herring, sprat or sandeels represent a major link between the base of the food web and top-predators such as harbour porpoise, grey seal, harbour seal, white-beaked dolphin and carnivorous fish, raising the need of an appropriate management of these small commercial fish species.

4.1. INTRODUCTION

The fertile waters of the North Sea represent a major life site for at least four different marine mammal species: the harbour porpoise, *Phocoena phocoena*, the harbour seal, *Phoca vitulina*, the grey seal, *Halichoerus grypus* and the white-beaked dolphin, *Lagenorhynchus albirostris* (Hammond et al., 1995). Harbour porpoise and harbour seal are the most common species in the Northeast Atlantic and the North Sea (Hammond et al., 1995; Boran et al., 1998). Their southern distribution seems to be limited to the Dutch Wadden Sea while white-beaked dolphins are generally concentrated in a band across the North Sea between 55° and 60°N, mostly to the West along the eastern British coast (Hammond et al., 1995; De Jong et al., 1999). Grey seal hauling and breeding sites are well known and described along the Northern UK coast (Nigel Bonner, 1989; Reijnders et al., 1995; OSPAR, 2000). However, some individuals have already been observed or are regularly found stranded in the Southern part of the North Sea suggesting more extended movements for these species (Haase, 1987; Leopold and Couperus, 1995; Abt et al., 2002; Jauniaux et al., 2002).

Other species like white-sided dolphins *Lagenorhynchus acutus*, sperm whales *Physeter macrocephalus* and fin whales *Balaenoptera physalus* can be occasionally sighted or found stranded, but are still considered very rare in the Southern North Sea (Camphuysen and Winter, 1995; Hammond *et al.*, 1995). This area is characterized by intricate systems of sand banks, mudflats, sandy islands and estuaries, obviously an unfavourable environment for such oceanic species (Hammond et al., 1995).

The distribution of marine mammals is strongly influenced by the distribution of their prey (Gowans and Whitehead, 1995; Gannon et al., 1997). However, despite regular and new observations in the Southern North Sea, very few data dealing with marine mammal diet within this area are available (Hislop, 1992; Santos et al., 1999). Strandings offer a good opportunity for scientists to collect biological data but in most cases, either stranded animal stomachs are empty, or digested material is not

suitable for diet research (Santos et al., 1994; Jauniaux et al., 2002). Moreover, strandings might represent potentially biased samples of animals as sick or injured animals may not be feeding normally prior to death (Sekiguchi et al., 1992; Santos et al., 1994).

The use of naturally occurring stable isotopes of carbon and nitrogen has provided complementary data to marine mammal feeding ecology (Hobson and Welch, 1992; Abend and Smith, 1995; Smith et al.,1996; Hobson et al., 1997; Burns et al., 1998; Hobson and Schell, 1998; Das et al., 2000). Indeed, the carbon and nitrogen isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in a consumer reflect those of its diet with a slight selective retention of the heavier isotope and excretion of the lighter one. As a result, these ratios (in delta notation: $\delta^{15}N$ and $\delta^{13}C$) typically show a trophic enrichment value of about 2-3‰ and 1‰ respectively (De Niro and Epstein, 1978; 1981; Hobson and Welch, 1992; Michener and Schell, 1994). Stable isotopes of nitrogen can be used to quantitatively assess the trophic level while the ^{13}C, rather than being a reliable indicator of trophic level, is generally used to indicate relative contributions to the diet of different potential primary sources in a trophic network, indicating for example the inshore *vs.* offshore, or pelagic *vs.* benthic contribution to food intake (Rau et al., 1992; Hobson et al., 1995; Smith et al., 1996; Lepoint et al., 2000).

Because stable isotope ratios in the tissue of a consumer are derived from assimilated food, the tissue reflects dietary input integrated over time, not just the last meal before stranding which might be considered as biased. The metabolic rates and the subsequent turn-over of elements differ between liver and muscle, allowing the evaluation of diet integrated between different periods of time (e.g., days, week or months; Tieszen et al., 1983; Hobson et al., 1996; 1997).

In order to contribute to the knowledge of their feeding ecology, $\delta^{15}N$ and $\delta^{13}C$ have been determined and compared in the livers and muscles of seven marine mammal species beached along the French Channel, Belgian and Dutch coasts between 1994 and 2000: the harbour porpoise, the harbour seal, the grey seal, the white-beaked-dolphin, the white-sided dolphin, the sperm whale and the fin whale. Stable isotope measurements have also been performed in 15 invertebrate and 34 fish species collected in the

Southern North Sea to delineate trophic relationships between marine mammals and other species from this area.

We have also addressed the question of whether more occasional species such as sperm whale, fin whale or white-sided dolphin actually feed within the North Sea Southern Bay such as commonly sighted species, namely the harbour porpoise, the harbour seal, the grey seal and the white-beaked dolphin.

4.2. MATERIAL AND METHODS

4.2.1. Marine mammal sampling

Liver and muscle of 46 harbour porpoises *Phocoena phocoena*, 23 harbour seals *Phoca vitulina*, 6 grey seals *Halichoerus grypus*, 7 white-beaked dolphins *Lagenorhynchus albirostris*, 2 white-sided dolphins *Lagenorhynchus acutus*, 7 sperm whales *Physeter macrocephalus* and 3 fin whales *Balaenoptera physalus* stranded on the French Channel, Belgian and Dutch coasts between 1994 and 2000 have been sampled and stored at –20°C until analysis (for necropsy details see Jauniaux et al., 1998; 2001; 2002).

4.2.2. Invertebrate and fish sampling

15 invertebrate and 34 fish species (species details in table 4.1) were collected with the Southern Part of the North Sea (between 51° and 56°N) during three cruises of the *R.V. Belgica* (Belgium) in September 2000, February and May 2001 and one cruise of the *Thalassa* (IFREMER, France) in March 2001 respectively. All samples were frozen and stored at –20°C until analysis.

4.2.3. Stable isotope measurements

Organisms may vary in their concentrations of lipids. As lipids have been shown to be depleted in ^{13}C relatively to the diet

(Tieszen *et al.*, 1983), they were extracted from samples using repeated rinses with 2:1 chloroform: methanol prior to analysis. After drying at 50°C (48h), samples were ground into a homogeneous powder. After grinding, samples containing inorganic carbonates were acidified with HCl (1N). As recommended by Pinnegar and Polunin (1999), when samples were acidified, $^{15}N/^{14}N$ ratios were measured before acidification due to significant modifications of these ratios after HCl treatment (Bunn et al., 1995).
Stable isotope measurements were performed on a V.G. Optima (Micromass) IR-MS coupled to a N-C-S elemental analyser (Carlo Erba) for automated analyses. Routine measurements are precise to within 0.3 $^{0}/_{00}$ for both 13-carbon and 15-nitrogen. Stable isotope ratios were expressed in δ notation according to the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.
Carbon and nitrogen ratios are expressed relative to the V-PDB (Vienna Peedee Belemnite) standard and to atmospheric nitrogen, respectively. Reference materials were IAEA-N1 ($\delta^{15}N$ = +0.4 ± 0.2‰) and IAEA CH-6 (sucrose) ($\delta^{13}C$ = -10.4 ± 0.2‰).
Isotopic model

Muscle $\delta^{15}N$ signatures of harbour porpoise, grey seal, harbour seal and white-beaked dolphin were converted to trophic position (TP) using the above equation (after Hobson and Welch, 1992; Lesage et al., 2001):

$$TP = 2 + (Dm - POM - TEF_{mmt})/ TEF$$

Where D_m = $\delta^{15}N$ value in marine mammal muscle, POM = $\delta^{15}N$ value of marine particulate organic matter of the Southern North Sea (fixed to 9‰ after Middelburg and Nieuwenhuize, 1998), and TEF = trophic enrichment factor in $\delta^{15}N$ for a specific tissue (Hobson and Welch, 1992). This latter value was set to a mean 3.4‰ for all community components (Lesage et al., 2001) except for marine mammals, for which a TEF value (TEF_{mmt}) of 2.4‰ was obtained in the muscles of 2 harbour seals fed on a constant herring diet (Hobson et al., 1996).

4.2.4. Data treatment

Parametric and non parametric tests were used to compare different groups: Kolmogorov-Smirnov test was used to assume the normality of the data. ANOVA followed by post-hoc multiple comparison tests (LSD test) have been used to compare the data between the different species. When the necessary assumptions to realise ANOVA were not gathered (normality of the variables and homogeneity of variances), Kruskall-Wallis was used followed by multiple comparisons based on the Kruskall-Wallis rank sums to test for pairwise differences among species. Mann-Whitney U-test was performed to compare differences among groups when variances were not homogenous. Spearman coefficient has been used to test correlations between the values. Results were judged significant when $p<0.05$.

4.3. RESULTS

Results on isotopic composition of invertebrates, fish and marine mammal species are summarized in table 4.1 and 4.2.

Table 4.1. Length (cm), $\delta^{13}C$ and $\delta^{15}N$ values (‰) and feeding type (FT) of selected invertebrates and fish of the Southern North Sea.

Values are given as mean ± SD in $^{0}/_{00}$. (Zof: Fish feeding on zooplankton, Ct: ctenophore (*: pool of 7 individuals), Omi: omnivorous invertebrates, Sf: suspension feeders, Gi: grazer invertebrates, Bifc: crustaceans feeding on benthic invertebrates, Mf: mollusc feeder, Bif: fish feeding on benthic invertebrates, Cf: carnivorous fish), nd: not determined. When length was not available weight is given between brackets.

Species		n	Length	$\delta^{13}C$	$\delta^{15}N$	FT
Ctenophores						
Pleurobrachia pileus	Sea Gooseberry	1*	nd	-12.9	16.6	Ct
Molluscs						
Buccinum undatum	Whelk	2	nd (37-45 g)	-15.1 ±0.2	13.7 ±0.1	Omi
Solen marginatus	Grooved razor shell	2	10.6-11.2	-16.6 ± 0.3	11.2 ± 0.1	Sf
Spisula solida	Thick trough shell	2	nd (9-11 g)	-17.4 ± 0.8	11.0 ± 1.2	Sf
Sepia officinalis	Common cuttlefish	5	nd (2.3-3.2 g)	-15.9 ± 0.6	16.1 ± 0.6	Bif
Loligo vulgaris	Common squid	9	nd (2.2-45 g)	-15.9 ± 0.6	17.2 ± 1.3	Bif
Crustaceans						
Crangon crangon	Common shrimp	1	nd (1.6-2.6 g)	-17.2	17.5	Bifc
Palaemon Cerratus	Common prawn	1	6-8	-15.8	14.6	Omi
Carcinus maenas	Common shore crab	3	nd (5-11 g)	-17.4 ± 0.3	15.5 ± 0.5	Omi
Liocarcinus holasatus	Swimming crab	2	nd (15-19 g)	-15.0 ± 0.1	16.1 ± 0.5	Omi
Pagurus berhardus	Hermit crab	2	nd (4.6-7.6 g)	-15.3 ± 0.6	15.0 ± 0.3	Omi
Echinoderms						
Asteria ruben	Common starfish	3	nd (46-182 g)	-13.8 ± 1.5	13.3 ± 0.5	Mf
Ophiura ophiura	Sand-star	1	nd (0.8 g)	-15.8	11.7	Sf
Echinorcardium cordatum	Sea potato	1	nd (22.6 g)	-17.4	10.6	Sf
Psammechinus miliaris	Sea urchin	1	nd (14.7 g)	-14.1	12.1	Gi

Table 4.1. (continued) : Length (cm), $\delta^{13}C$ and $\delta^{15}N$ values (‰) and feeding type (FT) of selected invertebrates and fish of the Southern North Sea.

Values are given as mean ± SD in ‰. (Zof: Fish feeding on zooplankton, Ct: ctenophore, Omi: omnivorous invertebrates, Sf: suspension feeders, Gi: grazer invertebrates, Bifc: crustaceans feeding on benthic invertebrates, Mf: mollusc feeder, Bif: fish feeding on benthic invertebrates, Cf: carnivorous fish), nd: not determined. When length was not available weight is given between brackets.

Species		n	Length	$\delta^{13}C$	$\delta^{15}N$	FT
Elasmobranchs						
Raja clavata	Thornback ray	5	37-94	-15.0 ± 0.6	14.9 ± 0.4	Bif
Raja montagui	Spotted ray	3	56-65	-16.6 ± 1.1	15.3 ± 0.7	Bif
Raja radiata	Starry ray	3	41-49	-16.5 ± 0.6	13.5 ± 0.2	Bif
Scyliorhinus canicula	Small spotted catshark	1	24-70	-15.6	18.2	Bif
Mustelus asterias	Stellate smooth-hund	2	70-81	-15.2 ± 0.13	16.2 ± 0.04	Bif
Clupeiformes						
Clupea harengus	Herring	3	10-30	-16.7 ± 0.1	14.4 ± 0.2	Zof
Engraulis encrasicolus	Anchovy	2	10-12	-16.8 ± 2	15.2 ± 0.2	Zof
Sprattus sprattus	Sprat	5	7-9	-17.3 ± 0.2	16.7 ± 0.5	Zof
Pleuronectiformes						
Buglossidium luteum	Solenette	1	4.5	-16.8	14.8	Bif
Limanda limanda	Dab	7	7-20	-15.9 ± 1.6	16.9 ± 0.5	Bif
Microstomus kitt	Lemon sole	1	16-42	-16.1	14.0	Bif
Platichthys flesus	Flounder	4	27-42	-16.9 ± 2.7	17.5 ± 1.9	Bif
Pleuronectes platessa	Plaice	2	19-31	-15.6 ± 0.4	16.9 ± 0.5	Bif
Solea (Pegusa)lascaris	Sand sole	5	10-12	-15.5 ± 0.5	17.4 ± 0.6	Bif
Solea solea (vulgaris)	Common sole	8	9-19	-15.7 ± 1.5	17.5 ± 0.9	Bif

Table 4.1. (continued) : Length (cm), $\delta^{13}C$ and $\delta^{15}N$ values (‰) and feeding type (FT) of selected invertebrates and fish of the Southern North Sea.

Values are given as mean ± SD in ‰. (Zof: Fish feeding on zooplankton, Ct: ctenophore, Omi: omnivorous invertebrates, Sf: suspension feeders, Gi: grazer invertebrates, Bifc: crustaceans feeding on benthic invertebrates, Mf: mollusc feeder, Bif: fish feeding on benthic invertebrates, Cf: carnivorous fish), nd: not determined. When length was not available weight is given between brackets.

Species		n	Length	$\delta^{13}C$	$\delta^{15}N$	FT
Scorpaeniformes						
Agonus cataphractus	Pogge	5	7-7.5	-16.4 ± 0.9	16.5 ± 0.4	Bif
Aspitriglia cuculus	Red gurnard	6	18-23	-15.8 ± 0.3	16.2 ± 0.5	Bif
Eutriglia gurnardus	Grey gurnard	5	7-21	-15.3 ± 0.7	16.7 ± 0.8	Cf
Liparis liparis	Common seasnail	5	7-9	-15.1 ± 0.4	17.6 ± 0.4	Cf
Trigla lucerna	Tub gurnard	1	26	-15.4	18	Cf
Perciformes						
Scomber scombrus	Mackerel	2	27-42	-17.0 ± 0.1	16.1 ± 0.1	Bif
Ammodytes tobianus	Lesser sandeel	5	15-19	-16.9 ± 1.5	15.9 ± 0.8	Zof
Hyperoplus lanceolatus	Greater sandeel	4	22-24	-16.0 ± 1.1	17.2 ± 0.4	Cf
Callionymus lyra	Common dragonet	5	13-18	-17.3 ± 0.5	17.0 ± 0.3	Bif
Mullus surmuletus	Striped red mullet	3	16-20	-16.3 ± 0.4	17.5 ± 0.3	Bif
Pomatoschistus sp	Goby	9	5-8	-17.1 ± 0.5	17.7 ± 0.8	Bif
Trachurus trachurus	Atlantic Horse mackerel	5	26-29	-16.3 ± 0.5	18.2 ± 0.8	Cf
Echiichtys vipera	Lesser weever	5	12-13	-16.3 ± 0.3	18.7 ± 0.2	Cf
Beloniformes						
Belone belone	Garfish	1	49	-15.9	18.0	Cf
Gadiformes						
Melanogrammus aeglefinus	Haddock	1	40	-16.9	14.8	Zof
Merlangius merlangus	Whiting	8	13-27	-16.3 ± 0.6	19.1 ± 0.7	Cf
Trisopterus luscus	Bib	5	15-17	-15.6 ± 0.4	19.1 ± 0.2	Cf
Gadus morhua	Cod	6	37-95	-16.3 ± 1.4	19.2 ± 1.4	Cf
Anguilliformes						
Anguilla anguilla	eel	1	38	-17.3	19.6	Cf

4.3.1. Stable isotopic composition of marine mammals

In the muscles, harbour seal, grey seal and white-beaked dolphin displayed similar mean $\delta^{15}N$ values, all significantly higher than that of harbour porpoise, sperm whale, white-sided-dolphin and fin whale (ANOVA, $p<0.002$, $F=2.0$, table 4.2, figure

4.1). $\delta^{15}N$ values were similar for harbour seal, grey seal and white beaked-dolphin (ANOVA, F=2.0, p>0.1). In turn, mean $\delta^{15}N$ values displayed by harbour porpoise were significantly higher than sperm whale, white-sided dolphin and fin whale (ANOVA, F=2.0, p<0.05). $\delta^{15}N$ did not differ significantly between white-sided dolphin and fin whale (ANOVA, F=2.0, p>0.1).

Table 4.2. $\delta^{13}C$ and $\delta^{15}N$ values in the muscles and livers of marine mammals collected along the Southern North Sea coast

Species	n	Muscle $\delta^{13}C$	Muscle $\delta^{15}N$	Liver $\delta^{13}C$	Liver $\delta^{15}N$
Fin whale *Balaenoptera physalus*	3	-18.5 ± 0.9	9.6 ± 1.3	-18.7 ± 0.4	11.3 ± 2.7
White-sided dolphin *Lagenorhynchus acutus*	2	-19.2 ± 0.1	10.8 ± 0.4	-17.8 ± 0.4	12.3 ± 0.4
Sperm whale *Physeter macrocephalus*	7	-19.0 ± 0.9	14.6 ± 0.6	-18.4 ± 0.3	13.2 ± 0.6
Harbour porpoise *Phocoena phocoena*	46	-16.4 ± 1.6	16.2 ± 1.6	-15.8 ± 1.6	16.7 ± 1.8
Grey seal *Halichoerus grypus*	6	-15.6 ± 1.6	17.9 ± 2.1	-15.3 ± 2.6	18.2 ± 0.9
Harbour seal *Phoca vitulina*	23	-16.2 ± 1.3	18.7 ± 2.5	-15.9 ± 1.1	17.9 ± 1.9
White-beaked dolphin *Lagenorhynchus albirostris*	7	-15.8 ± 0.7	18.8 ± 1.1	-15.6 ± 0.7	17.2 ± 2.1

Muscle mean $\delta^{13}C$ did not differ significantly between harbour porpoise, harbour seal, grey seal and white-beaked dolphin (ANOVA, F=1.0, p>0.1). Mean $\delta^{13}C$ values displayed by white-sided dolphin, sperm whale and fin whale did not differ significantly (ANOVA, F=1.0, p>0.5) but were significantly lower than those of other species (ANOVA, F=0.1, p<0.02).

Based on their muscle $\delta^{15}N$ values, trophic levels were estimated for harbour porpoise, grey seal, harbour seal and white-beaked dolphin (table 4.3). Trophic levels were not estimated for sperm whale, fin whale and white-sided dolphin as their depletion in $\delta^{13}C$ strongly suggested that they did not feed within this area (see discussion below).

In the livers, isotopic patterns were quite similar to muscles with harbour seal, grey seal and white-beaked dolphin displaying higher mean $\delta^{15}N$ values compared to other species (table 4.2, figure 4.1 and 4.3). $\delta^{15}N$ values were similar between harbour seal, grey seal and white-beaked dolphin (ANOVA, $F=1.5$, $p>0.5$). Harbour porpoise displayed significantly lower values than harbour seal (ANOVA, $F=1.5$, $p<0.02$) but its $\delta^{15}N$ values did not differ significantly from that of grey seal ($p=0.06$) or white-beaked dolphin ($p=0.5$). Hepatic $\delta^{15}N$ values were similar for white-sided dolphin, fin whale and sperm whale (ANOVA, $F= 1.5$, $p>0.5$) and were significantly lower than those of other species (ANOVA, $F=1.5$, $p<0.001$).

Hepatic $\delta^{13}C$ values were similar for harbour seal, grey seal, white-beaked dolphin and harbour porpoise (ANOVA, $F=1.4$, $p>0.5$). These $\delta^{13}C$ values were significantly higher than those displayed by white-sided dolphin, sperm whale and fin whale (ANOVA, $F=1.4$, $p<0.05$).

Hepatic compared to muscle $\delta^{13}C$ and $\delta^{15}N$ values were similar for harbour seal and grey seal (T-student test for dependant samples, $p>0.1$, figure 4.2 and 4.3). No tests was performed for white-sided dolphin and fin whale as only two liver samples were available. Muscle $\delta^{15}N$ values of white-beaked dolphin were significantly higher than hepatic ones (T-student for dependant samples, $p<0.005$) while $\delta^{13}C$ values were similar ($p>0.1$). Muscle $\delta^{15}N$ values displayed by sperm whale were significantly higher than hepatic ones (T-student for dependant samples, $p<0.02$). By contrast, hepatic mean $\delta^{13}C$ and $\delta^{15}N$ values of harbour porpoise were significantly higher than muscle values (T-Student, $p<0.01$ and $p=0.05$ respectively, figures 2 and 3). The two smallest porpoises (80 and 87 cm) displayed high $\delta^{15}N$ values (19.3 and 18.1‰ respectively) compared to adult females and males (figure 4.4). However, due to the small sample size, no statistical test was performed. For harbour porpoises, adult females displayed higher muscle $\delta^{15}N$ than adult males (figure 4.4, T-student for independent samples, $p<0.05$) while juvenile isotopic values were similar between sexes ($p>0.5$).

4.3.2. Fish and invertebrate muscle isotopic composition.

The highest mean $\delta^{15}N$ values was measured in the eel while the lowest mean value has been recorded in the echinoderm *Echinocardium cordatum* (table 4.1). The range of values was wide as individual $\delta^{15}N$ data ranged from 8.6‰ in the muscle of one fin whale to 21.9‰ in the muscle of one goby.

Based on their gut content composition and their lifestyle (personal observations, Greenstreet et al., 1997; Miller and Loates, 1997; Quéro and Vayne, 1997), the species were classified into feeding types (table 4.1). The majority of the macro- and megafaunal taxa investigated proved to be either zooplankton feeder invertebrates (Ct) such as the Ctenophore *Pleurobrachia pileus*, zooplanktivrous fish (Zof), suspension feeders (Sf), omnivorous invertebrates (crustaceans such as the common shore crab), crustaceans feeding on benthic invertebrates (Bifc), mollusc feeders (Mf), grazer invertebrates (Gi, mainly the sea urchin *Psammechinus miliaris*), fish feeding on benthic invertebrates (Bif) or carnivorous fish (Cf, mainly fish or squids feeders). Mean isotopic composition values had been calculated for each feeding type and compared to marine mammal muscle data (figure 4.1).

The suspension feeders displayed the lowest mean $\delta^{15}N$ value (mean: 11.1‰) followed by invertebrate grazers (mean: 12.1‰) and mollusc feeders (mean: 13.2 ‰). Carnivorous fish displayed higher mean $\delta^{15}N$ values than fish (and squids) feeding on benthic invertebrates, fish feeding on zooplankton and omnivorous invertebrates (figure 4.1, ANOVA, $F=2.0$, $p<0.001$). However, data did not differ significantly between carnivorous fish (mean: 17.8‰), crustaceans feeding on benthic invertebrates (mean: 17.3 ‰) or the Ctenophore *Pleurobrachia pileus* (mean: 16.6‰, ANOVA, $F= 2.0$, $p>0.1$). Omnivorous invertebrates were significantly enriched in ^{13}C compared to fish feeding on zooplankton (ANOVA, $F=1.0$, $p<0.0001$) while $\delta^{15}N$ data did not differ significantly between these two feeding groups.
The Ctenophore *Pleurobrachia pileus*, the grazer invertebrate, *Psammechinus miliaris* and the mollusc feeder *Asteria rubens* were considerably enriched in ^{13}C (mean $\delta^{13}C$ value: -12.9‰, -

14.1‰ and -13.8‰ respectively) compared to other feeding types (table 4.1, figure 4.1).

Herring sampling was performed during two cruises in September 2000 and in May 2001. Stable isotope data and length differed significantly between these two samplings: herrings caught in May were significantly longer and depleted in ^{13}C and ^{15}N compared to herrings caught in September (figure 4.5, T-student for independent samples, $p<0.001$).

4.3.3. Comparison with marine mammals

$\delta^{15}N$ values displayed by carnivorous fish did not differ significantly from that of grey seals or white-beaked dolphins (Mann-Whitney U-test, $p>0.1$, figure 4.1). However, carnivorous fish $\delta^{15}N$ data were significantly higher than that of harbour porpoises (Mann Whitney U-test, $p<0.001$) and lower than that of harbour seals (Mann-Whitney U test, $p<0.01$). Within the fish species, only the zooplankton feeders such as Clupeiforms or the lesser sandeel *Ammodytes tobianus* displayed lower mean $\delta^{15}N$ and $\delta^{13}C$ values than harbour porpoises (Mann- Whitney U test, $p<0.005$ and $p<0.0001$ respectively). Sperm whales, fin whales and white-sided dolphins were significantly depleted in ^{13}C compared to other feeding groups or marine mammals from the Southern North Sea (figure 4.1 and figure 4.2, Kruskall Wallis, $p<0.001$).

4.4. DISCUSSION

4.4.1. PATTERN OF CARBON ISOTOPE SIGNATURES

$\delta^{13}C$ signatures vary widely (range up to 9.4 ‰) among organisms collected within the Southern North Sea (table 4.1, figure 4.1).

Sperm whales, white-sided dolphins and fin whales are highly ^{13}C-depleted relative to the POM, invertebrate, fish and other marine mammal species (table 4.1, table 4.2, figure 4.1 and 4.2). This low $\delta^{13}C$ measured in the muscles and livers of sperm whales, white-sided dolphins and fin whales can be related to a more oceanic feeding.
Stable carbon isotope ratios have proven most useful in identifying where particular organisms feed as $\delta^{13}C$ values are typically higher in species from coastal or benthic food webs than in offshore food webs (Hobson, 1999; Lesage et al., 2001). No flesh has been found in the digestive tract of the sperm whales and this seems to indicate that the whales had not been feeding within the Southern North Sea prior to stranding (Jauniaux et al., 1998).

Male sperm whales are recorded as including a significant proportion of squids and fish in their diet in the deep waters of North Atlantic and Arctic waters (Santos et al., 1999). In the Northern hemisphere, they leave warm waters at the beginning of the summer to reach feeding grounds on the perimeter of the polar zone, returning again in winter (Santos et al., 1999). From our isotopic data, it appears that despite regular sightings of sperm whales within the Southern part of the North Sea, they do not feed mainly within this area, even on local cephalopods. Indeed, the squid species sampled in the Southern North sea as *Loligo vulgaris* or *Sepia officinalis* display higher $\delta^{13}C$ (and $\delta^{15}N$) than sperm whales (table 4.1 and 4.2). Oceanic or abyssal cephalopods have quite a different isotopic signature more similar to that of sperm whales (Ostrom et al., 1993; Abend and Smith, 1995; Iken et al., 2001). Similar conclusions can be drawn for fin whales and white-sided dolphins stranded along the Belgian and Dutch coasts. The depletion in ^{13}C observed for the two white-sided dolphins might also be linked to a more offshore feeding. $\delta^{13}C$ of these animals are similar to that recorded in other parts of the Northeast Atlantic, along the Irish coasts (see chapter 3).

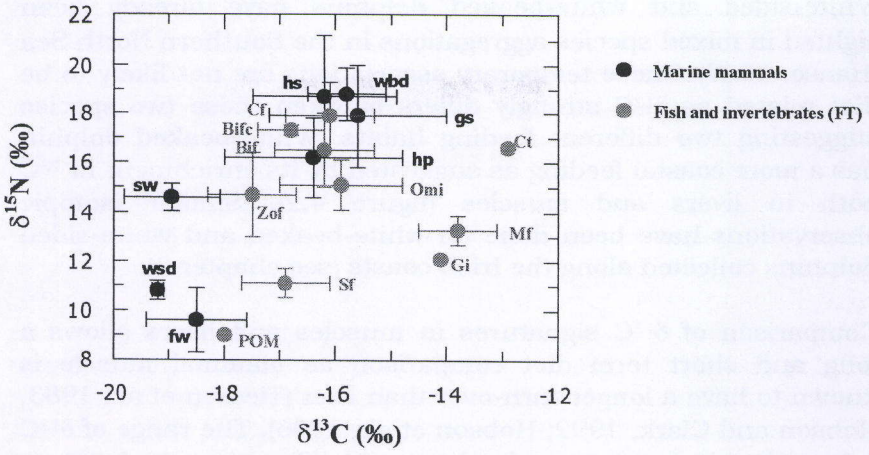

Figure 4.1. Muscle $\delta^{13}C$ and $\delta^{15}N$ mean values (± SD) of selected invertebrate, fish and marine mammals from the Southern North Sea

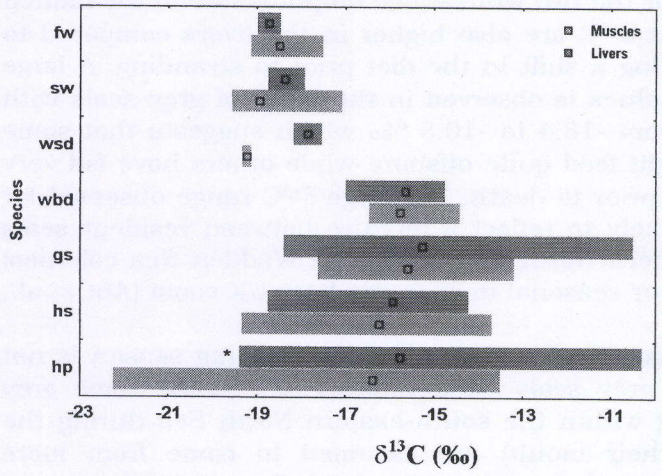

Figure 4.2. Mean and range of $\delta^{13}C$ values in the livers and muscles of marine mammals from the Southern North Sea coasts. *: $p<0.05$, T-test for dependant samples

fw: fin whale, **sw**: sperm whale, **wsd**: white-side dolphin, **wbd**: white-beaked dolphin, **gs**: grey seal, **hs**: harbour seal, **hp**: harbour porpoise, **Zof**: fish feeding on zooplankton, **Omi:** omnivorous invertebrates, **Sf**: suspension feeders, **Ct**: ctenophores, **Gi**: grazer invertebrates, **Bifc**: crustaceans feeding on benthic invertebrates, **Mf**: mollusc feeders, **Bif:** Fish and cephalopods feeding on benthic invertebrates, **Cf**: carnivorous fish, **POM:** particulate organic matter from the Southern North Sea (data from Middelburg and Nieuwenhuize, 1998).

White-sided and white-beaked dolphins have already been sighted in mixed species aggregations in the Southern North Sea (Haase, 1987). These temporary associations are not likely to be diet related as $\delta^{13}C$ strongly differs between these two species suggesting two different feeding habits. White-beaked dolphin has a more coastal feeding as suggested by its enrichment in ^{13}C both in livers and muscles (figure 4.2). Similar isotopic observations have been done on white-beaked and white-sided dolphins collected along the Irish coasts (see chapter 3).

Comparison of $\delta^{13}C$ signatures in muscles and livers allows a long and short term diet comparison as mammal muscle is known to have a longer turn-over than liver (Tieszen et al., 1983, Hobson and Clark, 1992; Hobson et al., 1996). The range of $\delta^{13}C$ values in marine mammals does not differ strongly between livers and muscles (figure 4.2). Harbour porpoises display higher $\delta^{13}C$ values in the livers suggesting that the animals were feeding closer inshore before stranding. No statistical test could be performed for the two white-sided dolphins due to the limited sample size but $\delta^{13}C$ are also higher in the livers compared to muscles reflecting a shift in the diet prior to stranding. A large range of $\delta^{13}C$ values is observed in the livers of grey seals with data ranging from -18.4 to -10.5 ‰ which suggests that some individuals might feed quite offshore while others have fed very near the coast prior to death. The large $\delta^{13}C$ range observed for grey seals is likely to reflect a mixture between resident seals from the Southern North Sea (likely the Wadden Sea colonies) and temporary or seasonal immigrants from UK coast (Abt et al., 2002).
Indeed, long-distance travel outside the breeding season is not uncommon in grey seals (McConnel et al., 1999). Some grey seals occurring within the south-eastern North Sea during the spring (after their moult) are assumed to come from more northern haul-out sites such as Scotland, Faroe Islands or from the Humber estuary, along the UK coasts (Abt et al., 2002). Resident grey seals are also increasingly observed during this last decade along the Wadden Sea coasts (Reijnders et al., 1995; Abt et al., 2002). The six grey seals from this study were collected along the Belgian and French Channel coasts between 2000 and 2001. No stranding has been recorded previously, at least for the Belgian coast. This apparent rate of increase could be related to the dispersing of the eastern UK stock into the south-eastern North Sea as observed seasonally in other areas (Abt et al., 2002).

Fish and invertebrates from the Southern North Sea are more enriched in ^{13}C compared to marine POM data from the Southern North Sea (Middelburg and Nieuwenhuize, 1998). Considerable overlap between species is observed (figure 4.1). Because $\delta^{13}C$ of an animal is largely determined by the $\delta^{13}C$ of its diet, these inter-taxa overlaps in isotope abundance indicate isotopic similarity among the respective diets of many of these species. As expected, suspension feeders are ^{13}C-enriched compared to POM. Among the different feeding types, the grazer invertebrates, the mollusc feeders and strikingly the Ctenophore are strongly ^{13}C -enriched. Deposit feeders have been shown to be more enriched in $\delta^{13}C$ compared to suspension feeders suggesting two different isotopic carbon signatures for suspended particulate matter and a mixture of suspended and sedimentary organic matter respectively (Dauby et al., 1998). Coastal or continental inputs are important within this area leading to ^{13}C enriched values for the particulate matter of the Channel and the North Sea compared to the Bay of Biscay (Dauby et al., 1994). However, the reason of the high enrichment of the ctenophore *Pleurobrachia pileus* is unclear. It strongly differs from other zooplanktivorous animals such as the herring or the lesser sandeel (table 4.1, figure 4.1).

$\delta^{13}C$ differs also between herring sampled in September 2000 and May 2001 (figure 4.5). Moreover, the mean length of these fish is higher in May than in September. The structure of herring stock in the Northeast Atlantic is complex with different sub-population, age and feeding regrouping (Jennings et al., 2001). Animals captured in May are adults displaying an oceanic carbon signature compared to animals collected in September which are juveniles with a typical coastal $\delta^{13}C$ enrichment compared to POM.

4.4.2. PATTERNS OF NITROGEN ISOTOPE SIGNATURES

Trophic levels of marine mammals

Trophic positions (TP) were estimated according to the model described by Lesage et al. (2001) for harbour porpoise, harbour seal, grey seal and white-beaked dolphin. TP were not evaluated for white-sided dolphins, fin whales and sperm whales. Indeed,

their ^{13}C-depletion strongly suggests that they feed out of the Southern North Sea (figure 4.1 and 4.2). A consumer isotopic signature is determined initially by the isotopic composition of the baseline phyto- and zooplankton sources which might vary widely with the sampling origin (Lesage et al., 2001; Riera et al., 1999; Middelburg and Nieuwenhuize, 1998). Southern North Sea POM values is not extrapolable to such oceanic species.

Within the North Sea, harbour seal, grey seal and white-beaked dolphin seem to occupy a similar trophic position at the top of the food web as suggested by their high $\delta^{15}N$ values in both muscles and livers (table 4.2 and 4.3 and figure 4.1).

Table 4.3. Trophic level comparison of harbour porpoise, grey seal, harbour seal and white-beaked dolphins (Pauly et al., 1998; this study)

Species	Pauly et al. (1998)	This study
Harbour porpoise *Phocoena phocoena*	4.1	**3.4**
Grey seal *Halichoerus grypus*	4.0	**3.9**
Harbour seal *Phoca vitulina*	4.0	**4.1**
White-beaked dolphin *Lagenorhynchus albirostris*	4.2	**4.2**

Estimated trophic levels calculated by Pauly et al. (1998), based on stomach content data indicate that trophic levels of harbour porpoise, harbour seal, grey seal and white-beaked dolphin are similar ranging between 4.0 and 4.2 (table 4.3). This extensive study has calculated a mean trophic level for each of the 97 marine mammal species and Pauly et al. (1998) have emphasized the tentative character of the modelling. TP estimated from $\delta^{15}N$ values are in good agreement with data described by Pauly et al. (1998) except for harbour porpoises which display a lower TP than the three other species (table 4.3). This discrepancy reflects a high proportion of prey displaying a low trophic level such as zooplanktivorous fish in the diet of harbour porpoise from the Southern North Sea. Furthermore, porpoises and dolphins are opportunist feeders taking advantage of local abundance of prey

(Lick, 1991; Rogan and Berrow, 1996; Couperus, 1997;Hassani et al., 1998).

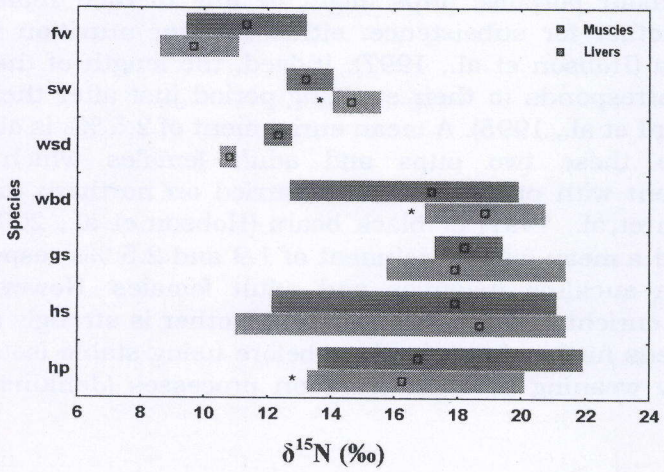

Figure 4.3. Mean and range of $\delta^{15}N$ values in the livers and muscles of marine mammals stranded along the French, Belgian and southern Dutch coasts. *: p<0.05, T-test for dependant samples

fw: fin whale, **sw**: sperm whale, **wsd**: white-side dolphin, **wbd**: white-beaked dolphin, **gs**: grey seal, **hs**: harbour seal, **hp**: harbour porpoise

Adult females porpoises feed at a higher trophic level than adult males while juvenile porpoises display no differences between sexes (figure 4.4). Previous studies have described that pregnant or lactating females might have a higher consumption, feed on larger prey or forage on different prey species (quoted by Aarefjord et al., 1995). The $\delta^{15}N$ high value encountered for the two harbour porpoise pups might be due to their reliance on their mother for subsistence, either milk or nutrition via the placenta (Hobson et al., 1997). Indeed, the length of these two pups corresponds to their suckling period just after their birth (Aarefjord et al., 1995). A mean enrichment of 2.5 ‰ is observed between these two pups and adult females which is in agreement with previous studies carried on northern fur seals (Hobson et al., 1997) or black bears (Hobson et al., 2000) who reported a mean $\delta^{15}N$ enrichment of 1.9 and 2.5 ‰ respectively between suckling juveniles and adult females. However this trophic enrichment between pup and mother is strongly specific and needs further investigations before using stable isotopes to quantify weaning or other lactation processes (Jenkins et al., 2001).

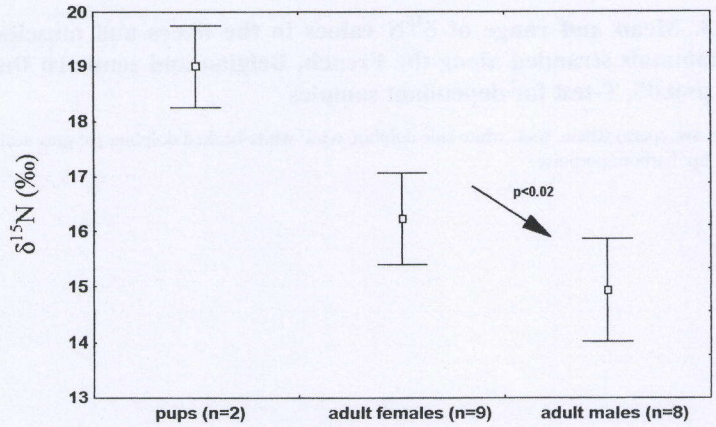

Figure 4.4: Muscle $\delta^{15}N$ (mean ± SD) in pups, adult male and adult female harbour porpoises. Number of individuals are given between brackets

Trophic relationships

Within the North Atlantic, herring, cod, sandeel, whiting goby and sole represent major prey for marine mammals with large intraspecific variations. Indeed, marine mammal diet has been shown to vary according to age of the individuals, abundance of prey species, seasons or geographic location (Evans, 1987; Lick, 1991; Pierce et al., 1991b; Aarefjord et al., 1995; Tollit et al., 1997; 1998).

In the North Sea, the harbour porpoise is known to feed on a wide range of pelagic and demersal fish species such as cod, herring, sole, goby or dab (Lick, 1991). Expressed as biomass of fish, sole and cod represented 41% and 25% respectively of the stomach content of harbour porpoises from German waters. In contrast, in the Baltic sea, cod can represent 70% of the harbour porpoise diet biomass. In young porpoises, goby is the main prey by number and weight (Lick, 1991). Harbour seals usually feed on clupeids, gadoids, cephalopods or sandeels depending on the prey avaibility. In the Wadden Sea, the harbour seal seems to prefer flatfish species (North Sea Task Force, 1993; Tollit et al.,1998). A large proportion of the grey seal diet includes sandeels (Ammodytidae), which can constitute over 70% of their diet depending on location and season (Prime and Hammond, 1990; Pierce et al., 1991a; Hammond et al., 1994).

Carnivorous fish such as Gadids display similar $\delta^{15}N$ to grey seals, harbour seals and white-beaked dolphins suggesting that they occupy a similar trophic level at the top of the food web (figure 4.1). Moreover, the mean values of fish species usually described as potential prey for North Sea marine mammals are high compared to harbour porpoise, harbour seal, grey seal and white beaked dolphin data (figure 4.5).

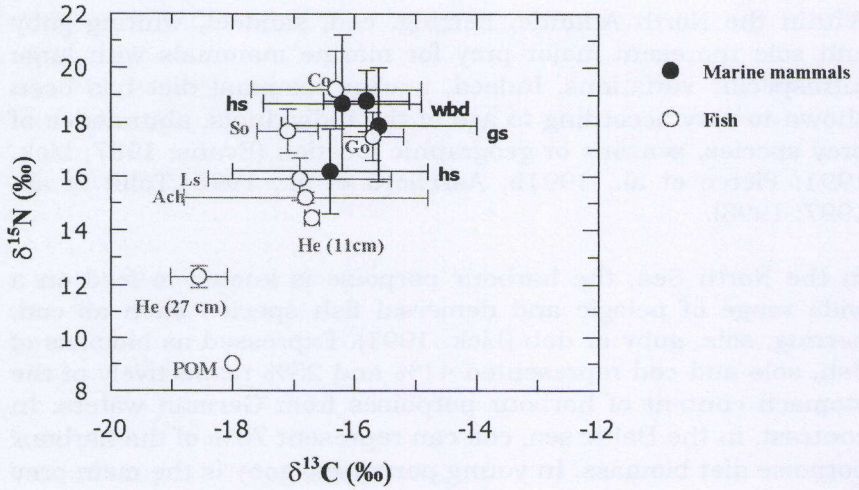

Figure 4.5: Muscle $\delta^{13}C$ and $\delta^{15}N$ mean values (± SD) in marine mammals and selected fish from the Southern North Sea.

fw: fin whale, **sw**: sperm whale, **wsd**: white-side dolphin, **wbd**: white-beaked dolphin, **gs**: grey seal, **hs**: harbour seal, **hp**: harbour porpoise **Co**: cod, **So**: sole, **Go**: goby, **Ls**: lesser sandeel, **Ach**: anchovy, **He**: herring, **POM**: particulate organic matter from the Southern North Sea (POM data from Middelburg and Nieuwenhuize, 1998).

As a $\delta^{15}N$ trophic enrichment of 2.4 ‰ is expected between potential prey and marine mammals (Hobson et al., 1996), usual described prey such as cod, other gadids, goby or sole are not likely to form the bulk of their diet. Indeed, mean $\delta^{15}N$ values of goby, sole or cod are higher than that of harbour porpoises. Gadids, goby and sole constituted a significant part of the diet of the North Sea harbour porpoise analysed by Lick (1991) but from isotopic data, they are not likely to constitute the main part of its diet within the Southern North Sea. Cod values are even higher than that of the two seal and white-beaked dolphin species (figure 4.4). However, a relationship between body size and $\delta^{15}N$ value has been shown for several marine species (Jennings et al., 2002) complicating the data interpretation. Even if the range of fish lengths are similar to that described for marine mammal preys (Aarefjord et al., 1995; Gannon et al., 1997), it is not excluded that smaller fish individuals with lower $\delta^{15}N$ values could be preyed by marine mammals.

On contrast, zooplanktivorous fish such as herring but also lesser sandeel or sprat, have lower $\delta^{15}N$ (and $\delta^{13}C$) values of about 2 to 4 ‰ and are likely to represent a major link between the base of the food web, which includes various bacterio, phyto- and zooplankton, and marine mammals or carnivorous fish (figure 4.1 and figure 4.5). Harbour and grey seals display higher trophic positions than harbour porpoises suggesting that higher trophic level fish such as lesser (or greater) sandeels may be preyed upon. Sandeel is one of the most common fish species on the continental shelf of the Northwest Europe, comprises 10 to 15% of the total fish biomass in the North Sea and is currently the target of the largest single species fishery in the North Sea (quoted by Rindorf et al., 2000). Sandeel avaibility has been shown to have major effects on breeding success of other marine predators such as seabirds (Rindorf et al., 2000). Mean $\delta^{13}C$ and $\delta^{15}N$ values are similar between grey and harbour seal suggesting that the range of prey species taken by these two seal foraging areas might overlap in this part of the North Sea. Similar observations based on faecal samplings have been performed in the south-western North Sea along the UK Wash coasts (Hall et al., 1998). Harbour seals are known to travel ten kilometres to feed (Thompson and Miller, 1990) whereas grey seals may travel far greater distances (Mc Connel et al., 1999). Additional partition of resources may result from differential foraging in offshore and coastal areas.

Our isotopic data clearly suggest that pleuronectiformes, cod and other gadids represent minor contributions to the diet of Southern North Sea marine mammals. The reason why these high trophic level fish do not represent a major part of marine mammal diet is unclear. The range of species preyed by marine mammals can be wide as more than 30 fish prey species can be identified in the diet of marine mammals (Lick, 1991). However, diet preferences seem to be oriented towards lower trophic level prey such as clupeids or sandeels. Previous studies indicate that during the past decades, intensive and size selective fishing has changed the size-structure of the North Sea fish community resulting in a general body size decrease. Smaller and early-maturing species have increased in relative abundance as suggest by the absolute and relative catches of smaller pelagic industrial species compared to large demersal species such as cod. (Fifth North Sea Conference Secretariat, 2002).

4.5. CONCLUSIONS

Despite occasional sightings of sperm whale, fin whale and white-sided dolphin in the Southern Bight of the North Sea, they mainly feed offshore *e.g.* within the North Atlantic. On the other hand, harbour porpoise, harbour seal, grey seal and white-beaked dolphin belong to the Southern North Sea food web. White-beaked dolphin, grey seal and harbour seal feed on higher trophic level prey than harbour porpoise but some diet overlap might occur between these species. Intraspecific variations have been observed in harbour porpoises: adult females display higher muscle $\delta^{15}N$ than adult males while no differences appear between juveniles. The lower trophic position occupied by harbour porpoise reflects a higher input in its diet of lower trophic level prey such as herring or sandeels which represent an important component in this ecosystem according to stable isotope values.

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CHAPTER 5: TRACE METALS IN MARINE MAMMALS FROM THE NORTH SEA AND ADJACENT AREAS:

Relation with stable carbon and nitrogen isotopes, sex, metallothioneins and nutritional status

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In preparation

ABSTRACT

In a first approach, trace metal concentrations were examined in 5 species of marine mammals found stranded along the Belgian and French Channel coast: the harbour porpoise *Phocoena phocoena*, the harbour seal *Phoca vitulina*, the grey seal *Halichoerus grypus*, the white-beaked dolphin *Lagenorhynchus albirostris* and the white-sided dolphin *Lagenorhynchus acutus*. Then, intraspecific sources of trace metal variations were investigated using the harbour porpoise as a model. Porpoises from the Belgian coasts were compared to individuals from the German North Sea and Baltic coasts, Denmark, Norway and Iceland. Toxicological results were confronted to most common pathological findings, namely as emaciation, lesions of the respiratory system or parasitism. Influence of diet through stable carbon and nitrogen analysis ($\delta^{13}C$ and $\delta^{15}N$), age and sex have also been considered.

Trace metal concentrations vary widely among species, especially Cd concentrations which are very high in the kidney of the two white-sided dolphins (88 $\mu g.g^{-1}$ dw for both individuals). For all marine mammal species, renal Cd concentrations were significantly correlated with muscle $\delta^{15}N$ meaning that Cd accumulation is partly diet related as a result of oceanic cephalopod ingestion. The nutritional status of stranded harbour porpoise from the Southern North Sea is poor compared to by-caught individuals from Norway and Iceland, as reflected by their blubber thickness, weight to length ratio and hepatic to total body mass ratio. The porpoises collected along the Southern North Sea coast display higher Zn and Hg concentrations compared to individuals collected in more preserved areas from the North Atlantic. Moreover, significant Zn, Hg and Se levels were observed with increasing emaciation severity. Porpoises displaying severe bronchopneumonia also have higher Zn concentrations probably due to the association previously described of emaciation and bronchopneumonia. These increasing concentrations are not related to a shrinking of liver mass as it remains strikingly unchanged during the emaciation process. As a result, hepatic trace metal load increases also. These observations tend to indicate a general redistribution of heavy metals within the organs (muscles to livers), which result from protein catabolism. Such a re-distribution could well be an additional stress for porpoises already experiencing stressful conditions (organochlorines, etc...). In contrast, Cu and Cd were never associated to emaciation. Other parameters such as age class or diet are more likely to be involved.

5.1. INTRODUCTION

Since 1998, the southern part of the North Sea including the Northern French, Belgian and Dutch coasts has been characterised by an increased number of stranded marine mammals such as the harbour porpoise, *Phocoena phocoena* (Jauniaux et al., 2002). These animals are final links in the Southern North Sea (see chapter 4) and therefore raise some concern about potential impact of pollutants on their health status. The question of whether environmental pollution may affect the dynamics and the health of marine mammal populations is unresolved (Harvell et al., 1999; Anan et al., 2002; Geraci and Lounsbury, 2002). Several investigations have been carried out in an attempt to evaluate organic contaminant effects at ambient environmental levels (*e.g.* Reijnders, 1986; Aguilar and Borrel, 1994; De Guise et al., 1995; de Swart et al., 1994; Jepson et al., 1999) while fewer studies have tried to link marine mammal health status and metal levels within the North Sea and adjacent areas (Hÿvarinen and Sipilä, 1984; Siebert et al., 1999; Bennet et al., 2001). Within the North Sea, heavy metals such as cadmium (Cd), mercury (Hg), or copper (Cu), remain substances for priority action under the OSPAR strategy with regards to hazardous substances (OSPAR, 2000). Chronic exposure to metals has been shown to cause immunosuppression or health deterioration in laboratory animals during controlled experimental condition (Krämer et al., 1993; Debacker et al., 2001;Wright and Welbourn, 2002). However, a clear cause and effects relationship between the residue levels of contaminants in marine mammals and the observed effects has only been demonstrated in only a few studies (Reijnders and Aguilar, 2002). The main reasons for the lack of proof of the impact of those pollutants in marine mammals are the difficulty of experimenting in laboratory conditions with these animals and the frequent occurrence of confounding factors that hamper the establishment of cause-effect relationships (Reijnders and Aguilar, 2002). An alternative to undesirable experimental manipulation on marine mammals is to carry out systematic post-mortem investigations to establish the health status of stranded and

by-caught animals coupled to systematic pollutants analysis (Jepson et al., 1999; Siebert et al., 1999; Bennet et al., 2001). We proposed here a further insight in the relationship between trace metal concentrations, species, diet, nutritional status and metallothioneins (MTs) using multiple stable isotope ($\delta^{13}C$ and $\delta^{15}N$), multiple tissue (liver, muscle and kidney) and multiple metal (Zn, Cu, Cd, Fe, Hg, Se) approach.

- In the **interspecific** part of this study, trace metals are compared between the tissues of harbour porpoise *Phocoena phocoena*, harbour seal *Phoca vitulina*, grey seal *Halichoerus grypus*, white-beaked dolphin *Lagenorhynchus albirostris* and white-sided dolphin *Lagenorhynchus acutus* found stranded along the Northern French and Belgian coasts, using previously reported $\delta^{13}C$ and $\delta^{15}N$ stable isotope measurements to infer specific diet influence on metal levels (see chapter 4). The use of stable isotopes in dietary studies is well established (Gannes et al., 1998; Kelly, 2000) and is based on the demonstration that stable isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in a consumer are related to those of their prey (De Niro and Epstein, 1978; 1981).

- **intraspecific** variations of metal concentrations and potential relationships with the health status are investigated in the harbour porpoise tissues by combining toxicological results, biometric data, stable isotope measurements and most common pathological findings, namely emaciation, parasitism, and lesions of the respiratory system, previously described (Siebert et al., 1999; 2001; Jauniaux et al., 2002). Harbour porpoises from the Southern North Sea are compared to those from the German North and Baltic Seas, Denmark, Iceland and Norway. Furthermore, the speciation of Zn, Cu, Cd and Hg on metallothioneins has been studied in the liver and kidney of selected porpoises from the Southern North Sea to get a furher insight on these protein role in metal homeostasis.

5.2. MATERIAL AND METHODS

5.2.1. SAMPLE COLLECTION AND STORAGE

49 harbour porpoises, 26 harbour seals, 6 grey seals, 2 white-sided dolphins and 9 white-beaked dolphins found stranded along the Belgian coastline and northern France have been sampled between December 1993 and May 2001 (tables 5.1 and 5.2). Moreover 75 harbour porpoises respectively collected along coasts of the German North Sea (11 stranded), German Baltic Sea (8 by-caught), Denmark (15 by-caught), Iceland (11 by-caught) and Norway (23 by-caught) have been added to this study (see table 5.2 for details). Post-mortem investigations were performed according to the protocol described by Kuiken and Garcia-Hartmann (1993). Samples of liver, kidney and muscle were taken and stored at –20°C until analyses. The nutritional status, the lesions of the respiratory tract and the parasitism were previously described by Siebert et al. (1999; 2001).

- The nutritional status (body condition) was judged on the basis of blubber thickness, measured at four different locations (sternal, caudodorsal, caudolateral and caudoventral to the dorsal fin) and on the state of muscles and divided into three categories: good, moderate, emaciated.

- The lesions of the respiratory system were grouped into mild (pulmonary oedema, pulmonary congestion, no nematode, no pneumonia), moderate (pulmonary oedema, pulmonary congestion, mild nematode infestation and mild pneumonia) and severe lesions (pulmonary oedema, pulmonary congestion, severe nematode infestation and severe pneumonia).

- Parasitism was divided into 4 broad categories: absence of parasitism, mild, moderate and severe based on observations during necropsy (Siebert, personal communication).

The ages of the porpoises were estimated by counting tooth growth layer groups (GLGs) according to Lockyer (1995).

5.2.2. ANALYTICAL METHODS

Zn, Cd, Fe and Cu analyses

After being weighed and dried for 48 h at 110°C, samples were digested with a solution of nitric acid (Merck 456) (1:3, v:v) and slowly heated to 100C° until complete digestion. Atomic absorption spectrophotometry (ARL 3510) was used to determine heavy metal concentrations (Cu, Zn, Cd, Fe). Concentrations are expressed as $\mu g.g^{-1}$ dry weight (dw).
Parallel to the samples, a set of certified material samples (CRM 278 Community Bureau of Reference, Commission of the European Communities) was also analysed to ensure the method's sensitivity. Recoveries ranged from 92 to 102 % for Cu, Zn, and Fe and 88% for Cd. Limits of detection were 0.01 $\mu g\ g^{-1}$ dw for Cu, 0.33 for Zn, 0.16 for Fe and 0.22 for Cd.

Total mercury analysis

The total mercury concentration was determined by specific atomic absorption spectrometry on a Perkin-Elmer Coleman Mas-50 Mercury analyser after the method described in detail in Joiris et al. (1991). Quality control included procedural blanks, replicate analyses and analyses of standard references tissue, using an external standard curve. The absolute detection limit is 10 ng Hg corresponding to 0.3 $\mu g.g^{-1}$ for an average 0.3 g dw sample.

Selenium analysis

Selenium was analysed by fluorimetry following complete digestion of the tissue by nitric, perchloric and hydrochloric acids, coupling to EDTA and 2,3-diaminonaphtalène and extraction by cyclohexane (modified from Mejuto Marti et al., 1987). The fluorimetre was set at excitation wavelength of 364nm and emission wavelength of 523nm with an emission slit of 2mm and an integration time for read mode of 2s.

Metallothionein analysis

3 to 4 grams of kidney were homogenized using an Ultra-Turrax in a 0.01 Mol ammonium formiate (pH = 7.4) containing 10 mM sodium azide and 0.01% dithithreitol and centrifuged at 26 000 g (60 min, 4°C). The supernatant was filtered on an AcA 54 at 4°C. Fractions were collected and absorbance profile read at 215, 254 and 280 nm using a spectrophotometer (Beckman DU 530).
After adding nitric acid (65%) to each fraction and to the pellet, homogenate and supernatant, all were slowly heated to 100°C until complete digestion. Samples were diluted using deionised water and filtered prior to heavy metal analysis (Zn, Cu and Cd) by atomic absorption spectrophotometry (ICPS: ARL 3510). Hg was analysed by flameless atomic absorption (Perkin-Elmer MAS-50A) as described above.

Stable isotope measurements

Organisms may vary in their concentrations of lipids. As lipids are in ^{13}C relatively to the diet (Tieszen et al., 1983), they were extracted from samples using repeated rinses with 2:1 chloroform : methanol prior to analysis. After drying at 50°C (48h), samples were ground into a homogeneous powder. Carbon dioxide and nitrogen gas were analysed on an V.G. Optima (Micromass) IR-MS coupled to a N-C-S elemental analyser (Carlo Erba). Routine measurements are precise to 0.3 $^{0}/_{00}$ for both 13-carbon and 15-nitrogen. Stable isotope ratios were expressed in δ notation according to the following:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.
Carbon and nitrogen ratios are expressed relative to the VPDB (Vienna Peedee Belemnite) standard and to atmospheric nitrogen, respectively.

Isotopic model

Muscle $\delta^{15}N$ signatures of harbour porpoises were converted to trophic position, TP, using the above equation (after Hobson and Welch, 1992; Lesage et al., 2001):

$$\mathbf{TP = 2 + (Dm - POM - TEF_{mmt})/ TEF}$$

Where D_m = $\delta^{15}N$ value in porpoise muscle, POM = $\delta^{15}N$ value of particulate organic matter (fixed to 9‰ after Middelburg and Nieuwenhuize (1998) for Southern North Sea marine particulate organic matter), and TEF = trophic enrichment factor in $\delta^{15}N$ for a particulate tissue (Hobson and Welch, 1992). This latter value was set to a mean 3.4‰ for all community components (Lesage et al., 2001) except for marine mammals, for which a TEF value (TEF_{mmt}) of 2.4‰ was obtained in the muscles of 2 harbour seals fed on a constant herring diet (Hobson et al., 1996).

5.2.3. DATA TREATMENT

Kolmogorov-Smirnov test was used to assume the normality of the data. When not distributed normally the variables were log-transformed to normalize their distribution. Multivariate analysis of variance (n-way MANOVA) followed by post-hoc multiple comparison tests (LSD test) have been used to compare the data between the different groups. Parametric Spearman- and non parametric Pearson- coefficients have been used to test correlations between the values. Results were judged significant when $p<0.05$ unless otherwise stated.
Comparisons between species as well as comparison between locations were made using the different tissues available: liver, kidney and muscle for heavy metals and liver and muscle for stable isotope ratios. Effect of species on heavy metals and stable isotope ratios were tested using a multivariate analysis of variance (one way-MANOVA). For Hg and Cd, multifactorial analysis of covariance (ANCOVA/MANCOVA) was used to adjust the effects for age (covariate).
Effects of emaciation (good, moderate, emaciated), lesions of the respiratory system (absence, mild, moderate, severe) and parasitism (absence, mild, moderate, severe) on trace metal

concentrations have been tested simultaneously (three-way MANOVA) on the liver, kidney and muscle of the porpoises from the German Baltic Sea, the German North Sea, the Danish, Norway and Icelandic coast, excluding porpoises from the Belgian and Northern France coasts. For the latter, only the effect of emaciation (one-way MANOVA) was judged, as data for the respiratory system and parasitism were not comparable to other locations.

5.3. RESULTS

5.3.1. INTERSPECIES COMPARISON

Hepatic, renal and muscle metal levels were compared between harbour porpoises, harbour and grey seals and white-beaked dolphins found stranded along the Northern French and Belgian coasts (tables 5.1 and 5.3). Main statistical results are summarised in the table 5.5. Only two white-sided dolphins were available and so, were excluded from the statistical analysis.

Table 5.1. Trace element concentrations ($\mu g.g^{-1}$ dry weight) in the liver, kidney and muscle of marine mammal species stranded along the Belgian and Northern French coasts expressed as a mean ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; nd: non determined

		Harbour seal	Grey seal	White-beaked dolphin	White-sided dolphin
Zn	liver	122 ± 58 (48-296) n=26	125 ± 21 (91-153) n=6	155 ± 103 (78-382) n=9	167 ± 25 (149-185) n=2
	kidney	108 ± 46 (67-268) n=24	86 ± 15 (67-111) n=6	90 ± 32 (26-119) n=9	118 ± 19 (105-131) n=2
	muscle	136 ± 62 (53-271) n=27	109 ± 23 (79-143) n=6	56 ± 16 (28-95) n=9	28 ± 6 (24-32) n=2
Cd	liver	0.1 ± 0.2 (< 0.05-0.9) n=26	<0.05 (<0.05-0.1) n=6	0.1 ± 0.1 (< 0.05-0.3) n=9	25 ± 4 (22-28) n=2
	kidney	1.0 ± 3 (<0.05-12) n=24	0.3 (0.05-0.6) n=6	0.5 ± 0.5 (<0.05-1.3) n=9	88 ± 0.3 (88.0-88.4) n=2
	muscle	<0.05 (<0.05-0.7) n=27	<0.05 (<0.05-0.3) n=6	0.4 ± 1.2 (<0.05-4) n=9	0.3 ± 0.03 (0.25-0.3) n=2
Fe	liver	2726 ± 2088 (413-8162) n=26	1439 ± 761 (739-2886) n=6	1411 ± 638 (683-2377) n=9	1215 ± 54 (1176-1253) n=2
	kidney	726 ± 220 (306-1237) n=23	534 ± 145 (299-721) n=6	458 ± 101 (336-608) n=9	757 ± 183 (627-886) n=2
	muscle	785 ± 266 (400-1387) n=27	559 ± 203 (214-803) n=6	612± 164 (302-880) n=9	734 ± 153 (625-842) n=2

Table 5.1 (continued). Trace element concentrations (µg.g^{-1} dry weight) in the liver, kidney and muscle of marine mammal species stranded along the Belgian and Northern French coasts expressed as a mean ± standard deviation, range of concentrations (minimum-maximum); n: number of samples; nd: non determined

		Harbour seal	Grey seal	White-beaked dolphin	White-sided dolphin
Cu	liver	21 ± 22 (5-118) n=26	37 ± 18 (7-57) n=6	26 ± 7 (10-36) n= 9	27 ± 6 (22-31) n=2
	kidney	20 ± 13 (9-74) n=24	12.5 ± 3.5 (7-15) n=6	11 ± 5 (4-19) n=9	13 ± 1 (13-14) n=2
	muscle	8 ± 3 (4-15) n= 27	6 ± 1.2 (4-7) n=6	8 ± 3 (3-14) n=8	5 ± 2 (4-7) n=2
Se	liver	8 ± 7 (1-30) n=22	8 ± 6 (4-14) n=3	51 ± 65 (1-157) n = 7	23 ± 12 (14-31) n=2
	kidney	5 ± 4 (0.7-13) n=20	10 ± 3 (7-13) n=3	3 ± 3 (0.2-9) n = 8	4 ± 4 (1-7) n=2
	muscle	1.5 ± 0.5 (1-2.2) n=8	2 ± 0.7 (1.5- 2.8) n=3	1.7 ± 0.1 (1.5-2.0) n=3	3 n=1
Hg	liver	7 ± 5 (1-19) n=14	8 ± 11 (0.8-24) n=4	74 ± 94 (7-140) n=2	138 ± 114 (56-219) n=2
	kidney	5 ± 5 (1-16) n=11	8 ± 4 (5-11) n=2	4.5 ± 4 (2-7) n=2	10 n=1
	muscle	3 ± 4 (0.9-14) n=11	3 ± 1.9 (1.5-4.2) n=2	4 ± 4 (0.9-7) n=2	4 ± 0.5 (3.4-4.1) n=2

- **Zn** differed significantly between species (one way-MANOVA, $F_{9,180}$=6.7 p<0.0001). These differences were driven by hepatic and muscle Zn concentrations while renal Zn concentrations did not differ significantly (table 5.5). Specifically, harbour porpoise hepatic Zn concentrations were significantly higher than those of harbour seal (post-hoc LSD test, p<0.0005). The highest muscle Zn level was found in harbour seal, significantly higher than that of harbour porpoise (post-hoc LSD test, p<0.00001) and white-beaked dolphin (post-hoc LSD test, p<0.00001).

- **Cd** levels differed between species (one-way MANOVA, $F_{6,100}$=5.5, p<0.0001). Differences appeared both in liver and

kidney (table 5.5). Muscle concentration were below or close to the detection limit and were excluded from statistical tests.

Very high Cd levels were found in the liver and kidney of the two white-sided dolphins (table 5.1). Harbour porpoises, harbour and grey seals and white-beaked dolphins displayed far lower concentrations. Among these four species, harbour porpoise displayed the highest mean hepatic and renal concentrations while grey seal displayed the lowest, close to the detection limit.

- **Fe** concentrations differed between species (one-way-MANOVA, $F_{9,180}=3.1$, $p<0.005$). Specifically, differences were driven by hepatic levels (table 5.5). The highest mean hepatic concentrations was found in the harbour seal, significantly higher than that of harbour porpoise (post-hoc LSD test, $p<0.0005$) and white-beaked dolphin (post-hoc LSD test, $p<0.05$).

- **Cu** concentrations differed significantly between species (one-way-MANOVA, $F_{9,177}=4.8$, $p<0.00001$). Differences were driven by hepatic and renal concentrations while muscle Cu concentrations remained similar between species (table 5.5). Specifically, harbour seal displayed higher hepatic Cu concentrations than grey seal (post-hoc LSD test, $p<0.05$) or harbour porpoise (post-hoc LSD test, $p<0.0005$). The highest renal Cu concentration was observed for the harbour seal and harbour porpoise and, both were significantly higher than that of white-beaked dolphin (post-hoc LSD test, respectively $p<0.005$ and $p<0.05$). Grey seal displayed also significantly lower renal Cu concentrations than harbour seal (post-hoc LSD test, $p<0.05$).

- **Se and Hg** concentrations remained similar between species in the liver, kidney and muscle. However, the interpretation of the results was weakened by the absence of age data for the two seal and dolphin species.

- **Relationship with stable isotopes**

Considering the whole data together (harbour porpoises, harbour seals, grey seals, white-beaked and white-sided dolphins), a significant relationship was observed between muscle $\delta^{15}N$ measurements previously reported (see chapter 4) and hepatic (Bravais-Pearson correlation, $r=-0.65$,

p<0.0001, n=64) and renal (Bravais-Pearson correlation, r=-0.65, p<0.0001, n=62) Cd (log-normalised) concentrations (figure 5.1). No relationship was observed with other metals.

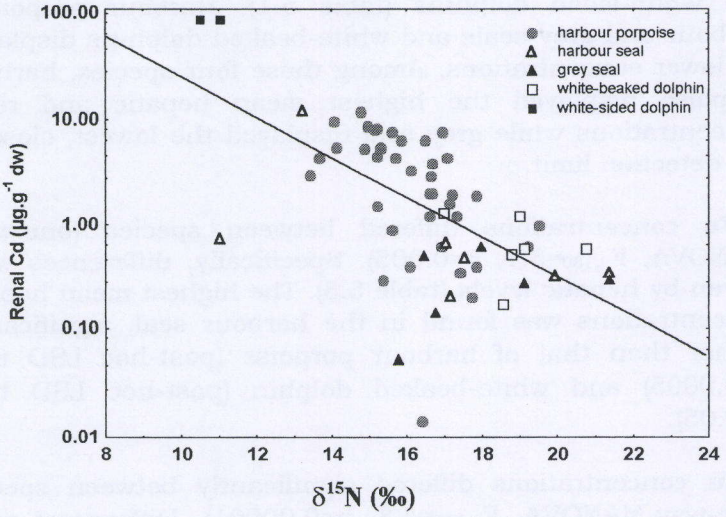

Figure 5.1. Relationship between muscle $\delta^{15}N$ and renal Cd concentrations of marine mammals stranded along the Belgian and neighbouring coasts using a log-scale

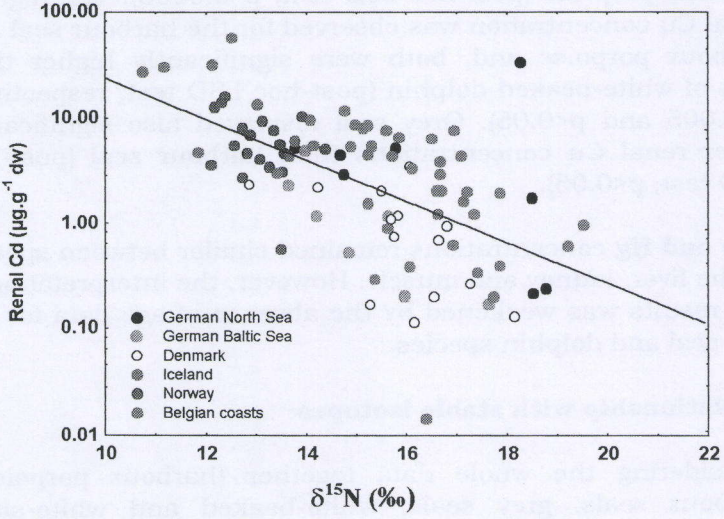

Figure 5.2. Relationship between muscle $\delta^{15}N$ and renal Cd concentrations of harbour porpoises from the Northeast Atlantic using a log-scale

5.3.2. INTRASPECIFIC VARIATIONS: THE CASE OF THE HARBOUR PORPOISE

These results included porpoises from the Belgian and Northern French coasts, which were compared to porpoises from the coasts of the German North and Baltic Seas, Denmark, Norway and Iceland (tables 5.2 and 5. 3).

Table 5.2. Sampling description of harbour porpoises from the North Sea and adjacent areas (2 juvenile porpoises were not evaluated for nutritional status).

	stranded	by-caught	sex		nutritional status		
			male	female	non-emaciated	moderate	emaciated
3 foetuses	1	2	1	2	3 (100%)	0	0
11 neonates	10	1	6	5	5 (46%)	3 (27%)	3 (27%)
82 juveniles	43	39	36	46	46 (56%)	7 (8.5%)	27 (33%)
38 adults	20	18	19	19	18 (47%)	8 (21%)	12 (32%)
TOTAL: 135	**74**	**61**	**63**	**72**	**73 (54%)**	**18 (13%)**	**42 (41%)**

Considering all the porpoises together, hepatic Zn and Cu concentrations differed between the foetuses, neonates (<90 cm), juveniles (<130 cm) and adults (>130cm) (1-way MANOVA, all effects $p<0.0001$, $F_{6,234}=12.6$ and 22.6). Specifically, foetuses displayed significantly higher hepatic Cu concentrations than neonates (post-hoc LSD tests $p<0.04$) which in turn had higher Cu concentrations than juveniles and adults (post-hoc LSD test, $p<0.0001$). Neonates displayed significantly lower Zn concentrations than foetuses (post-hoc LSD test , $p=0.05$), juveniles (post-hoc LSD test $p<0.007$) and adults (post-hoc LSD test $p<0.01$). Juvenile and adult porpoises displayed similar Cu and Zn concentrations (post-hoc LSD test $p>0.6$). For other statistical tests, the 3 foetuses and 11 neonates will be excluded.

Table 5.3. Trace element concentrations (mean ± SD, range and number of samples) in harbour porpoises from different North Sea and Atlantic areas

		Belgian and Northern France coasts	North Sea German coasts	Baltic Sea German coasts	North Sea Danish coasts	Iceland coasts	Norwegian coasts
Zn	liver	234 ± 179 (40-684) n=49	217± 175 (71-727) n=15	135 ± 56 (78-242) n=9	117 ± 44 (74-261) n=17	135 ± 26 (114-187) n=11	111 ± 47 (69-248) n=23
	kidney	107 ± 27 (68-201) n=48	106 ± 26 (69-157) n=13	90 ± 20 (68-136) n=9	92 ± 23 (74-166) n=15	101 ± 16 (78-120) n=11	96 ± 25 (73-164) n=22
	muscle	74 ± 33 (24-193) n=51	83 ± 27 (51-134) n=14	58 ± 27 (27-124) n=9	71 ± 34 (39-148) n=15	44 ± 13 (20-61)) n=10	53± 40 (19-163) n=23
Cd	liver	0.5 ± 0.6 (<0.05-2.5) n = 49	0.6 ± 1.3 (< 0.05-5) n=15	0.2 ± 0.2 (< 0.05-0.5) n=9	0.2 ± 0.1 (<0.05-0.4) n=17	6 ± 11 (0.4-39) n=11	0.4 ± 0.5 (<0.05-2) n=23
	kidney	3.1 ± 3.1 (<0.05-12) n=48	4 ± 9 (<0.05-33) n=13	1.1 ± 1.5 (<0.05-5) n=9	1.1 ± 1.0 (0.1-3.5) n=15	19 ± 17 (2-58) n=11	6 ± 4.5 (<0.05-16) n=22
	muscle	<0.05 (<0.05-0.2) n=51	<0.05 (<0.05-0.2) n=14	<0.05 (<0.05) n=9	0.1 ± 0.3 (<0.05-1.2) n=15	0.1 ± 0.15 (<0.05-0.4) n=10	<0.05 (<0.05-0.8) n=23
Fe	liver	1435 ± 995 (324-4490) n = 49	1182 ± 735 (235-2556) n=15	962 ± 461 (349-1826) n=9	1287 ± 573 (527-2588) n=17	1567 ± 544 (697-2378) n=11	1249 ± 300 (792-1774) n=23
	kidney	621 ± 375 (267-2539) n=48	518 ± 209 (219-844) n=13	914± 1091 (425-3808) n=9	750 ± 183 (541-1203) n=15	819 ± 255 (511-1368) n=11	815 ± 250 (489-1681) n=22
	muscle	752 ± 246 (262-1204) n=51	638 ± 250 (260-1168) n=14	467 ± 193 (225-802) n=9	544 ± 112 (327-671) n=15	651 ± 143 (373-842) n=10	563 ± 163 (256-887) n=23

Table 5.3 (continued). Trace element concentrations in harbour porpoises from different North Sea and Atlantic areas

		Belgian and Northern France coasts	North Sea German coasts	Baltic Sea German coasts	North Sea Danish coasts	Iceland coasts	Norwegian coasts
Cu	liver	39 ± 38 (9-257) n=49	64 ± 53 (20-169) n=15	62 ± 77 (18-260) n= 9	28 ± 16 (15-88) n=17	30 ± 11 (13-53) n=11	48 ± 61 (12-217) n=22
	kidney	17 ± 11 (7-73) n= 48	15 ± 2 (11-19) n=13	16 ± 3 (13-21) n=9	15 ± 6 (11-36) n=15	16 ± 4 (11-23) n=11	16 ± 4.5 (10-32) n=22
	muscle	7 ± 4 (2-22) n = 51	7 ± 2 (3-11) n= 14	7± 2 (4-10) n=9	5 ± 1.3 (3-8) n=15	5 ± 1.5 (1.8-7) n=10	6 ± 2 (2-9) n=23
Se	liver	14 ± 21 (0.6-99) n=37	11 ± 11 (1-39) n=13	6 ± 3 (2-10) n = 8	9 ± 7 (3-32) n=14	10 ± 8.5 (2.5-26) n=6	13 ± 7 (4-34) n=23
	kidney	6 ±4 (1-21) n=34	nd	nd	nd	nd	17 ± 9 (3-33) n=21
	muscle	3.8 ± 8.5 (0.4-39) n=20	nd	nd	nd	nd	5 ± 15 (0.2-71) n=22
Hg	liver	80 ± 279 (0.6-1681) n=37	13 ± 17 (1-56) n=15	4.5 ± 3.6 (0.9-12) n=9	22 ± 36 (1-147) n=17	16 ± 14 (1.4-44) n=11	14 ± 10 (1-32) n=22
	kidney	8 ± 13 (0.9-42) n=18	nd	nd	nd	nd	6 ± 9 (1-43) n=21
	muscle	5 ± 7 (0.7-28) n=17	nd	nd	nd	nd	9 ± 31 (0-146) n=22

Zn. Geographic location of collections significantly affected Zn concentrations (1-way MANOVA all effects $F_{15,281}=4.68$, $p<0.00001$; figure 5.3), specifically in liver and in muscle while renal Zn remained similar (table 5.5). Hepatic Zn concentrations did not differ significantly between porpoises from Belgian and German parts of the North Sea, which in turn were significantly higher than those from German Baltic Sea (post-hoc LSD test, $p=0.05$ and $p<0.05$ for Belgian and German porpoises respectively), Denmark (post-hoc LSD test, $p<0.005$) and Norway (post-hoc LSD test, $p<0.0005$).

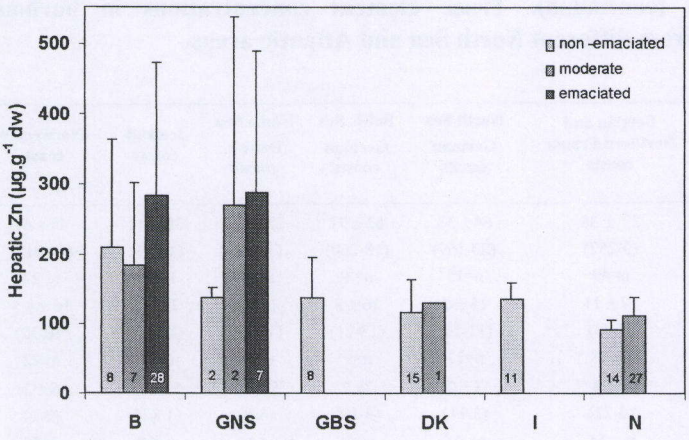

Figure 5.3. Hepatic Zn concentration in relation to the nutritional status and sampling location considered (B: Belgian and French coasts; GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway. The 3 foetuses and 11 neonates are excluded)

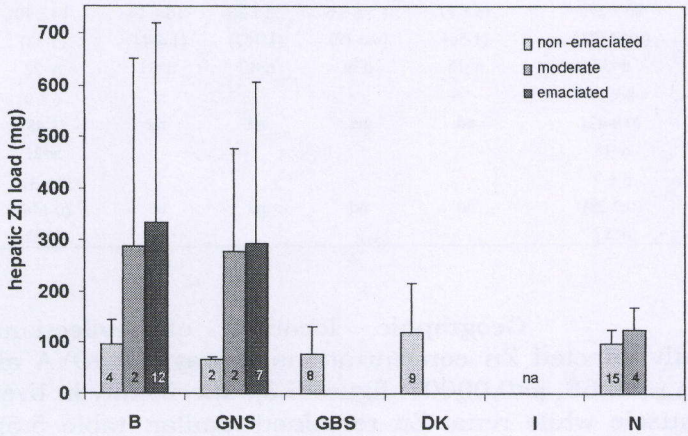

Figure 5.4. Hepatic Zn load in relation to the nutritional status and sampling location considered (B: Belgian and French coasts; GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway. The 3 foetuses and 11 neonates are excluded)

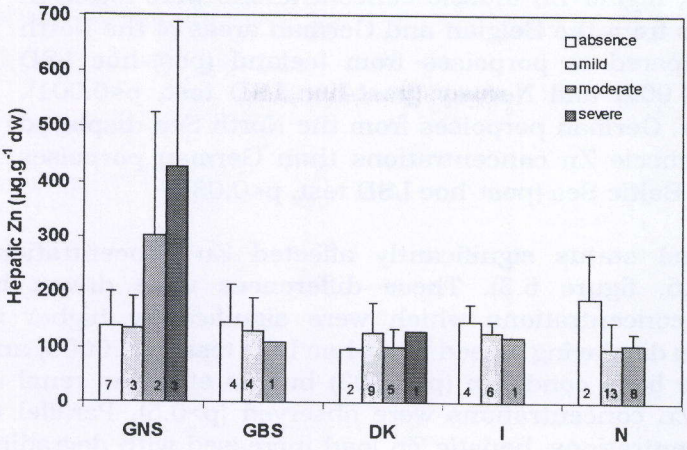

Figure 5.5. Hepatic Zn concentration in relation to the lesions of the respiratory system and sampling location considered (GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway. The 3 foetuses and 11 neonates are excluded)

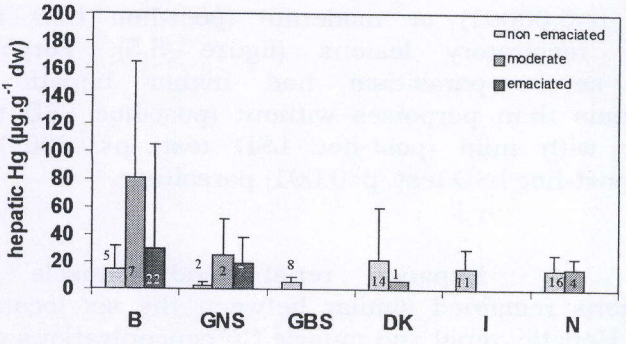

Figure 5.6. Hepatic Hg concentration in relation to the nutritional status and sampling location considered (B: Belgian and French coasts GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway. The 3 foetuses and 11 neonates are excluded)

Similarly, higher Zn muscle concentrations were found in porpoises from the Belgian and German areas of the North Sea compared to porpoises from Iceland (post-hoc LSD test, $p<0.002$) and Norway (post-hoc LSD test, $p<0.001$). Moreover, German porpoises from the North Sea displayed higher muscle Zn concentrations than German porpoises from the Baltic Sea (post-hoc LSD test, $p<0.03$).

Nutritional status significantly affected Zn concentrations (table 5.5, figure 5.3). These differences were driven by hepatic concentrations which were significantly higher in porpoises displaying a good (post-hoc LSD test, $p<0.0006$) and moderate body condition ($p<0.003$) but no effect on renal or muscle Zn concentrations were observed ($p>0.5$). Parallel to the concentrations, hepatic Zn load increased with degrading body condition (figure 5.4).

Lesions of the respiratory system and parasitism significantly affected Zn concentrations (table 5.5, $F_{9,107}= 3.4$, $p<0.0015$ and $F_{3,44}=4.9$, $p<0.005$ respectively). Porpoises displaying severe lesions of the respiratory system had significantly higher hepatic Zn concentrations than porpoises without (post-hoc LSD test, $p<0.00005$), or displaying mild (post-hoc LSD test, $p<0.00001$) or moderate (post-hoc LSD test, $p<0.00003$) respiratory lesions (figure 5.5). Porpoises displaying severe parasitism had higher hepatic Zn concentrations than porpoises without (post-hoc LSD test, $p<0.01$) or with mild (post-hoc LSD test, $p<0.0001$) or moderate (post-hoc LSD test, $p<0.001$) parasitism.

Cu. Hepatic, renal and muscle Cu concentrations remained similar between the six locations (table 5.5). Hepatic, renal and muscle Cu concentrations were not affected by the nutritional status or lesions of the respiratory system. The degree of parasitism (absence, mild, moderate or severe) affected significantly the Cu concentration. Porpoises displaying mild or moderate parasitism display lower hepatic and muscle concentration than porpoises without parasitism.

Cd. Geographic location of collections significantly affected hepatic and renal cadmium

concentrations adjusted for age (table 5.3 and 5.5, 1-way MANCOVA adjusted effects, $R_{10,122}=7.5$, $p<0.0001$). Specifically, hepatic and renal concentrations in porpoises from Iceland were significantly higher than those from other locations (post-hoc LSD test, $p<0.02$). The lowest Cd concentrations were found in the liver and kidney of porpoises from the German Baltic Sea and Danish coasts. These two locations did not differ significantly between them (post-hoc LSD test, $p>0.5$). Porpoises from Norway, German North Sea and Belgian coasts display similar hepatic and renal Cd concentrations (post-hoc LSD test, $p>0.5$), in an intermediate position between German Baltic and Danish porpoises (post-hoc LSD test, $p<0.05$) and Icelandic porpoises (post-hoc LSD test, $p<0.02$). No statistical test was performed in muscle as the concentrations were below or close to the detection limit.
The nutritional status, lesions of the respiratory system and parasitism did not affect hepatic or renal Cd concentrations in porpoises ($p>0.5$).

Fe. Muscle Fe concentrations differed significantly between sampling locations while hepatic and renal concentrations did not (table 5.5). Renal Fe concentrations were related to the nutritional status. Specifically, porpoises in good body condition display significantly higher renal Fe than porpoises displaying a moderate (post-hoc LSD test, $p<0.02$) or emaciated body condition (post-hoc LSD test, $p<0.002$).

Renal Fe concentrations are also related to lesions of the respiratory system as porpoises without bronchopneumonia displayed higher renal Fe concentrations than porpoises characterized by mild (post-hoc LSD test, $p<0.05$), moderate (post-hoc LSD test, $p<0.05$) or severe (post-hoc LSD test, $p<0.0005$) lesions. No relationship between parasitism and hepatic, renal or muscle Fe concentration was observed.

Hg. Geographic location affected hepatic Hg concentrations standardised for age (1-way ANCOVA, all effects, $F_{5,76}=2.48$, $p<0.04$, figure 5.6).

Porpoises from Belgian and French coasts carried the highest Hg concentrations, significantly higher than porpoises from

the German Baltic Sea (post-hoc LSD test, $p=0.005$). One six-year-old emaciated female porpoise displayed an hepatic Hg concentration of 1681 $\mu g.g^{-1}$ dw (table 5.3) but this exceptionally high concentration was excluded from statistical comparison to avoid potential biased results. Considering all the sampling locations simultaneously, neither nutritional status or lesions of the respiratory system were related to the hepatic mercury concentrations (table 5.5). However, considering only Belgian and German North Sea porpoises together, Hg tended to increase with degrading body condition (one-way ANCOVA, adjusted effects for age, $p<0.07$).

Harbour porpoises displaying moderate or severe parasitism have higher mercury concentration than porpoise without or with mild parasitism (3-way ANCOVA, $F_{3,45}=2.8$, $p<0.05$, adjusted effects for age).

Se. Se concentrations did not differ significantly between sampling locations (one-way ANCOVA, $F_{5,63}=0.7$, $p>0.6$, adjusted effects for age). Neither body condition ($F_{2,38}=1$, $p>0.3$), lesions of the respiratory system ($F_{3,38}=1.2$, $p>0.2$) or parasitism ($F_{3,38}=2.31$, $p>0.09$) affected Se concentrations (three-way ANCOVA, adjusted effect for age). However, considering only Belgian and German North Sea porpoises together, hepatic Se tended to increase with degrading body condition (one-way ANCOVA, adjusted effects for age, $p<0.002$). Se was significantly correlated with Hg concentrations in each country except for Iceland porpoises (Pearson product-moment correlation, $r=0.64$, $n=6$, $p<0.2$) for which the small sample size might have weakened the test.

Metallothioneins. Hepatic MTs bound 51% of the total zinc and 48% of the total Cd while Hg is detected mainly in the insoluble fraction of the tissue (table 5.4). Increase of Zn concentration in the liver was followed by an increase of the % of Zn bound to MTs ($r=0.8$, $p=0.0002$) while the percentage of Zn bound to hepatic high molecular weight proteins decreases significantly ($r=-0.73$, $p=0.01$, figure 5.7).

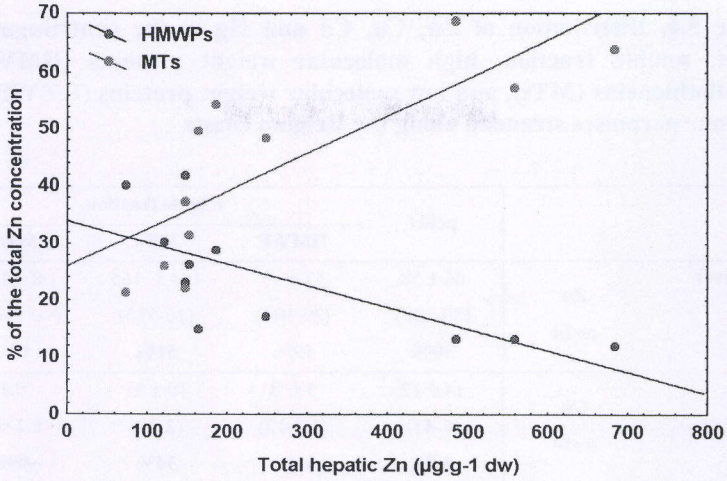

Figure 5.7. Relationship between the total hepatic Zn concentration and the fraction bound to metallothioneins (MTS) and high molecular weight proteins (HMWPs)

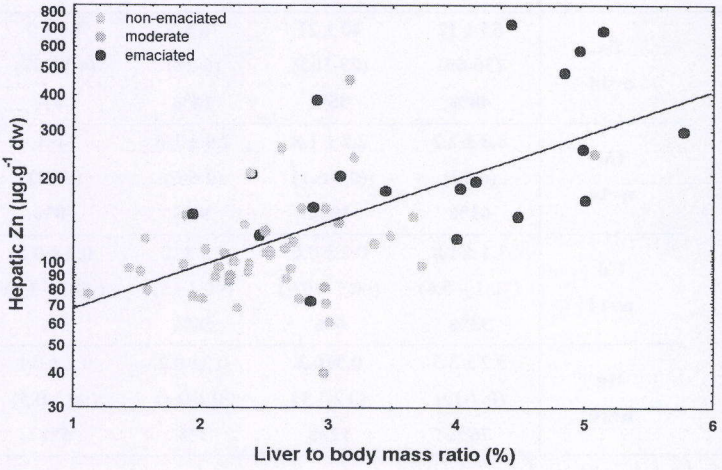

Figure 5.8. Relationship between the ratio liver to total body mass and zinc concentrations using log-scale

Table 5.4. Distribution of Zn, Cu, Cd and Hg in the centrifugation pellet, soluble fraction, high molecular weight proteins (HMWP), metallothioneins (MTs), and low molecular weight proteins (LMWP) of harbour porpoises stranded along the Belgian coasts

		pellet	soluble fraction		
			HMWP	MTs	LMWP
Liver	**Zn n=14**	86 ± 58 (30-228) **30%**	53 ± 19 (29-104) **19%**	144 ± 165 (16-595) **51%**	1.8 ± 117 (<0.1-6,2) **~0%**
	Cu n=14	14 ± 12 (6-43) **48%**	5 ± 3 (1.3-12) **18%**	10 ± 11 (2-39) **34%**	<0.1 (<0.1-6.1) **~0%**
	Cd n=14	0.2 ± 0.2 (0.01-0.6) **9%**	0.5 ± 0.6 (<0.1-1.8) **21.5%**	1.1 ± 0.8 (0.1-2.7) **48%**	0.5 ± 1 (<0.1-3.4) **21.5%**
	Hg n=14	43 ± 51 (0.5-167) **99.5%**	0.1 ± 0.1 (<0.1-0.3) **~0.25%**	<0.1 (<0.1-0.2) **~0.25%**	<0.1 (<0.1) **~0%**
kidney	**Zn n=14**	55 ± 12 (36-69) **48%**	40 ± 21 (23-105) **35%**	16 ± 5 (6-28) **14%**	4.4 ± 7 (<0.1-27) **<4%**
	Cu n=14	8.8 ± 2.2 (6-13) **61%**	2.8 ± 1.8 (0.4-6.1) **19%**	2.9 ± 1.6 (0.6-6) **20%**	<0.1 (<0.1) **~0%**
	Cd n=14	1.1 ± 1,8 (<0,1 – 5,6) **33%**	0.2 ± 0,6 (<0,1 – 0,9) **6%**	1.9 ± 2 (<0,1 - 5) **56%**	0.2 ± 0,8 (<0,1 – 1,7) **5%**
	Hg n=10	3.2 ± 3.3 (0.7-12) **76%**	0.5±0.3 (0.2-1.1) **12%**	0.3± 0.2 (0.1-0.4) **7%**	0.2 ± 0.1 (<0.1-0.5) **5%**

$\delta^{13}C$ and $\delta^{15}N$ measurements. Geographic location significantly affected $\delta^{13}C$ and $\delta^{15}N$ measurements (figure 5.9). Porpoises from the German Baltic North Sea displayed significantly higher $\delta^{15}N$ values than porpoises from Belgian coasts (post-hoc LSD test $p<0.02$), Denmark (post-hoc LSD test $p<0.01$), German Baltic Sea (post-hoc LSD test $p<0.005$), Norway (post-hoc LSD test $p<0.0001$) and Iceland (post-hoc LSD test $p<0.0001$). Porpoises from Belgian coasts were significantly enriched in ^{13}C compared to individuals from Denmark (post-hoc LSD test $p<0.005$), German Baltic Sea (post-hoc LSD test $p<0.0005$), Norway (post-hoc LSD test $p<0.0001$) and Iceland (post-hoc LSD test $p<0.005$).

Muscle $\delta^{13}C$ and $\delta^{15}N$ (figure 5.13) were compared between harbour porpoises from Belgian and German coasts of the North Sea displaying a good, moderate and emaciated body condition. Stable isotope ratios remained similar between these three different categories (table 5.5).

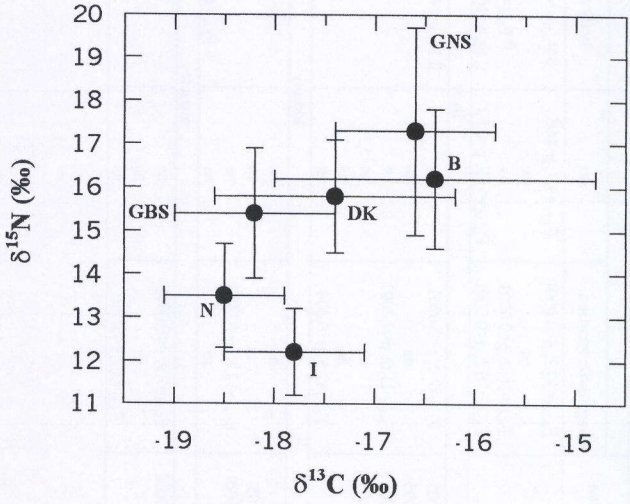

Figure 5.9. Muscle $\delta^{13}C$ and $\delta^{15}N$ values of harbour porpoises from the North Sea and adjacent areas

B: Belgian and French coasts (see chapter 4); GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway

Table 5.5. Influence of species, age, body condition, lesions of the respiratory system and parasitism on metal concentrations in the tissues of the harbour porpoise. The symbols ⇑ or ⇓ indicate an increasing or decreasing trend; ns not significant; nd: not determined. <LD: below the detection limit so not included in the statistical treatment; log: indicate the data were log-transformed before statistical treatment to ensure a normal distribution

Variable	species comparison	harbour porpoise					
		geographic location	sex	body condition	Lesions of the respiratory system	parasitism	age (log)
Blubber thickness	nd	$F_{5,103}$=29.3; p<0.0001	$F_{1,65}$=9.7, p<005	⇓ $F_{2,31}$=14.5, p<0.001	⇓ $F_{3,31}$=3.8, p<0.05	ns	r=0.3, p>0.005
Liver mass	nd	ns	ns	ns	ns	ns	r=0.5, p<0.0001
Liver to body mass (%)	nd	$F_{4,65}$=10.7; p<0.0001	ns	⇑ $F_{2,31}$=6, p<0.01	⇑ $F_{3,31}$=5, p<0.01	⇑ $F_{3,31}$=5.1, p<0.01	ns
Weight to length ratio	nd	$F_{5,112}$=16.5, p<0.0001	$F_{1,116}$=6.49, p<0.02	⇑ $F_{2,31}$=10.3, p<0.0005	⇓ $F_{3,31}$=5, p<0.01	ns	r=-0.65, p<0.0001
liver							
Zn (log)	$F_{3,76}$=5.1, p<0.005	$F_{5,95}$=9.2; p<0.0001	ns	⇑ $F_{2,44}$=14.4, p<0.0001	⇑ $F_{3,44}$=9, p<0.0001	⇑ $F_{3,44}$=5, p<0.005	ns
Cu (log)	$F_{3,76}$=5.3, p<0.005	ns	ns	ns	ns	⇓ $F_{3,42}$=3.9, p<0.05	r=-0.45, p<0.0001
Cd (log)	$F_{3,51}$=5.5, p<0.005	$F_{5,61}$=11.6; p<0.0001	ns	ns	ns	nd	r=-0.3, p<0.01
Fe	$F_{3,76}$=5.2, p<0.005	ns	ns	ns	ns	ns	ns
Se (log)	ns	ns	ns	ns	ns	ns	r=0.47, p<0.0001
Hg (log)	ns	$F_{5,76}$=2.5, p<0.004	ns	ns	ns	⇑ $F_{3,45}$=2.8, p<0.05	r=0.37, p=0.001
kidney							
Zn (log)	ns	ns	ns	ns	ns	ns	ns
Cu (log)	$F_{3,76}$=5.3, p<0.05	ns	ns	ns	ns	ns	r=-0.3, p<0.005
Cd (log)	$F_{3,51}$=12.8, p<0.0001	$F_{5,61}$=12.8, p<0.0001	ns	ns	ns	ns	r=0.3, p<0.005
Fe (log)	ns	ns	ns	⇓ $F_{2,44}$=6.3, p<0.005	⇓ $F_{3,44}$=4.8, p=0.005	ns	ns
muscle							
Zn (log)	$F_{3,76}$=17.1, p<0.0001	$F_{5,95}$=9.9; p<0.0001	ns	ns	ns	ns	r=-0.37, p<0.001
Cu (log)	ns	ns	ns	ns	ns	⇓ $F_{3,42}$=4.1, p<0.05	r=-0.4, p<0.0001
Fe	ns	$F_{5,95}$=3.7, p<0.005	ns	ns	ns	ns	ns

5.4. DISCUSSION

5.4.1. INTERSPECIES VARIATIONS

Strong interspecific differences were observed for the different marine mammal species collected along the Belgian coast (tables 5.1 and 5.5). Zn, Cu, Fe and Cd differed significantly between species while Hg and Se did not. However, the interpretation of the results was weakened by the small sample size and absence of age data for the two seal and dolphin species, especially for metals displaying an age accumulation such as Cd, Se or Hg (reviewed by Das et al., 2002). Strikingly, the highest Cd concentrations were observed for the two adult white-sided dolphins (table 5.1). This mean concentration of 88 $\mu g.g^{-1}$ dw is much higher than that described for one adult white-sided dolphin collected along the Irish coast (19 $\mu g.g^{-1}$ dry weight; see chapter 3) and for 2 adult individuals from the U.S. Atlantic coasts (about 2 $\mu g.g^{-1}$ dry weight, Kuehl et al., 1994). In the Southern North Sea, higher Cd concentration were only described in livers and kidney of 5 sperm whales found stranded along the Belgian and Dutch coast in 1995 (Holsbeek et al., 1999). Both sperm whale and white-sided dolphin were depleted in $\delta^{13}C$ compared to other trophic components of the Southern North Sea indicating that they fed mainly on pelagic prey (see chapter 4). Previous studies have recorded cephalopods as the most important prey category for sperm whales (Santos et al., 1999). Moreover, the high Cd level encountered in these two species can be related to oceanic cephalopods as these invertebrates have been shown to concentrate Cd in their digestive glands (Bustamante et al., 1998). This observation is enhanced by the overall decreasing relationship found between $\delta^{15}N$ and Cd (log-) concentrations in livers and kidneys of harbour porpoises and other marine mammal species from this study (figures 5.1 and 5.2). Such a relationship has been previously described (see chapter 3). Our results confirm that high Cd values encountered in some marine mammal species are partly diet related as a result of an ingestion of prey displaying typical low $\delta^{15}N$ and $\delta^{13}C$ values and high Cd levels such as oceanic cephalopods

(Ostrom et al., 1993; Bustamante et al., 1998;, Hooker et al., 2001).

Hg concentrations were higher in the 2 white-beaked dolphins compared to the two seal species whereas they display similar $\delta^{15}N$ values (see chapter 4). This is likely an age-effect as the harbour seals of this study are juveniles. A Hg concentration of 229 $\mu g.g^{-1}$ dw has been reported in the liver of one white-beaked dolphin found stranded along the French Channel coast (see chapter 3). Moreover, no relationship has been observed between hepatic and muscle $\delta^{15}N$ and mercury concentrations which have already been reported in a previous study (see chapter 3). Mercury levels in marine mammals are strongly influenced by age and the high Hg concentrations found in the studied marine mammals probably reflect a contamination over the animal's whole life rather than a biomagnification process.

BODY CONDITION OF HARBOUR PORPOISES

Seasonal changes in body fat condition in relation to food consumption have already been observed in captive and free-living harbour porpoises (Kastelein et al., 1997; Lockyer et al., 2001). These seasonal changes of total body mass and blubber thickness are correlated with water temperature in relation with the functional role of blubber as insulation and energy reserves (Iverson, 2002). However, the blubber thickness decrease can also be linked to emaciation characterized by a moderate to severe amyotrophy (Siebert et al., 2001; Jauniaux et al., 2002). Indeed, the mean blubber thickness increases from the Southern North Sea to the Northern Norway or Iceland and as expected, decreases drastically with increasing emaciation severity (figure 5.10).

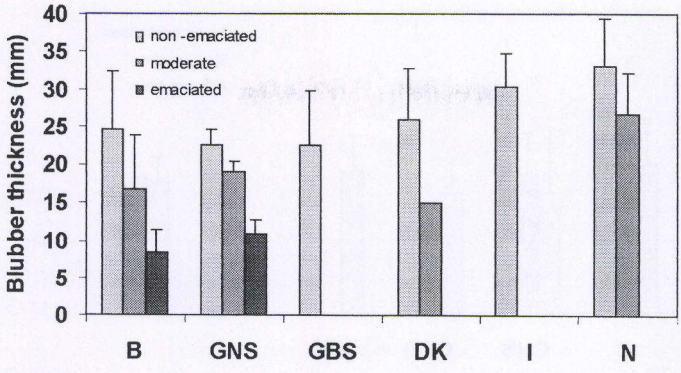

Figure 5.10. Blubber thickness (mm) of harbour porpoises in relation to the nutritional status and sampling location considered. B: Belgian and French coasts; GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway

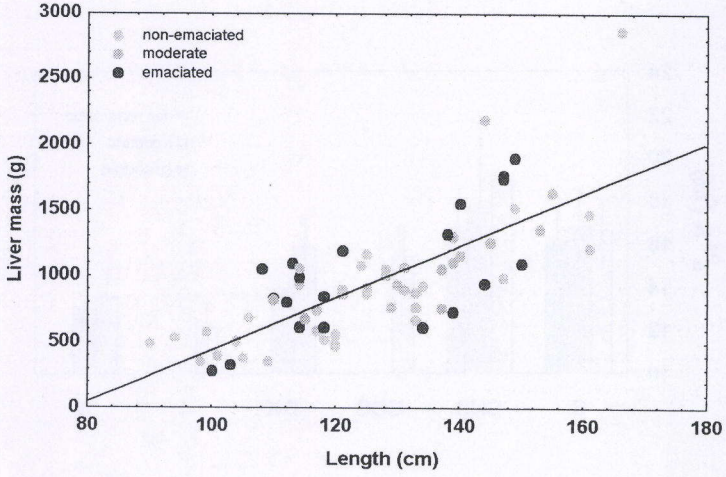

Figure 5.11. Relationship between the length and liver mass of the harbour porpoise in relation to the nutritional status

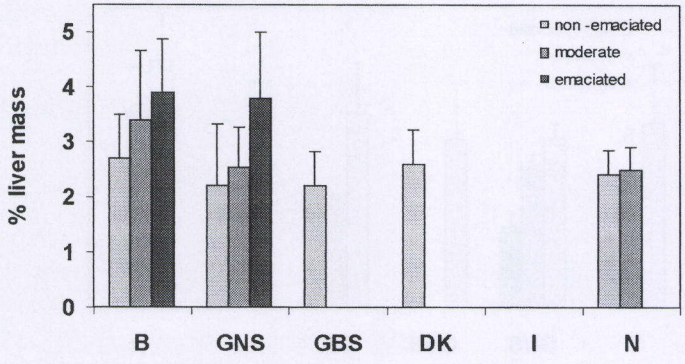

Figure 5.12. Liver to total body mass (%) of harbour porpoises in relation the nutritional status and sampling location considered. B: Belgian and French coasts; GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway

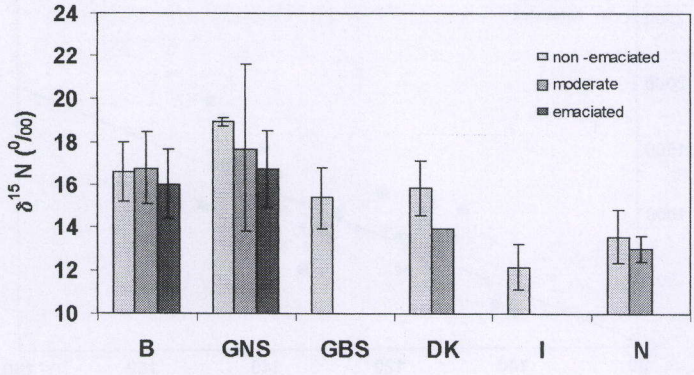

Figure 5.13. Muscle $\delta^{15}N$ of harbour porpoises in relation to the nutritional status and sampling location considered B: Belgian and French coasts; GNS: German North Sea coasts; GBS: German Baltic coasts; DK: Denmark, I: Iceland; N: Norway

Due to these geographical variations, blubber thickness alone is a poor indicator of the nutritional status of the harbour porpoise. Bennet et al. (2001) used the body mass to body length ratio to estimate nutritional status. However, this factor varies with the age of the individual especially during their growth. In mammals and birds, fasting results in a significant reduction of body and liver masses (Krämer et al., 1993; Debacker et al., 2000; 2002). Liver mass was reduced of 2/3 in experimentally emaciated rats compared to controls (Hashimoto et al., 1998), resulting from glycogenolysis and protein catabolism (Krämer et al., 1993). The liver mass of the harbour porpoises increases with the length of the individuals but remains strikingly unchanged between porpoises in good or moderate body condition, or emaciated (figure 5.11).

This means that during the starvation process, lipids of the blubber are preferentially metabolized (Markussen, 1995). The ratio of the liver to the total body mass increases significantly with degrading body condition (figure 5.12) as a result of the loss of total body mass. In contrast to total body mass, this ratio is independent of length or age and so appears as a complementary continuous datum to characterize body condition. The poor nutritional status of the porpoises has been previously related to a reduced food intake linked to parasitism (Siebert et al., 1999), ulceration of the upper gastro-intestinal tract or bronchopneumonia (Jauniaux et al., 2002).

TOXICOLOGY

Short-term fasting or prolonged starvation, through mobilization of body reserves, are known to influence the metabolism of trace elements such as Zn or Cu (Filteau and Woodward, 1982; Spencer et al., 1985; Eisler et al., 1993; Krämer et al., 1993; Debacker et al., 2000; 2001). Previous studies mentioned higher metal levels associated with stranded or debilitated birds and marine mammals (Siebert et al., 1999; Debacker et al., 2000; Bennet et al., 2001). The results of the present study further confirm these results and, more important, clearly points out a general rise of Zn and Hg

in the livers of harbour porpoises as the emaciation worsens: the more the harbour porpoises are emaciated, the higher Zn and Hg levels in their tissues (figures 5.3, 5.6, 5.8) while renal Fe decreases in relation to emaciation. Cu and Cd levels in tissues do not seem to be influenced by the body condition (table 5.5). In contrast to previous studies (Debacker et al., 2000; 2001; Krämer et al., 1993), the increasing concentrations are not linked to the loss of the individual liver mass which remains constant throughout the whole emaciation process (figures 5.11 and 5.12).

Zn absorption from the intestine can increase in states of malnutrition (Spencer et al., 1985). Furthermore, a large proportion of the muscle mass is lost during emaciation and Zn bound to muscle metalloproteins can be redistributed, after proteolysis, in the plasma and other tissues such as the liver (Spencer et al., 1985; Krämer et al., 1993).

However, heavy metals such as Zn and Hg may lead to a decrease of the total body mass in various wildstock or laboratory animals (Eisler, 1993; Debacker et al., 2001). Zn exerts also a critical influence on mammalian immune system (Salgueiro et al., 2000; Rink and Gabriel, 2001). Adverse biochemical effects of a high Zn intake on Cu metabolism have been reported (Spencer et al., 1985, Fosmire, 1990). A high Zn intake may cause substantial change of the Zn/Cu ratio in various tissues. In marine mammals, Zn is generally correlated with Cu as a result of both an antagonistic behavior and binding to metallothioneins. This relationship has been observed for the by-caught porpoises from Norway but not for the porpoises found stranded along the Southern North Sea coast reflecting a severe homeostasis disturbance (figure 5.14).

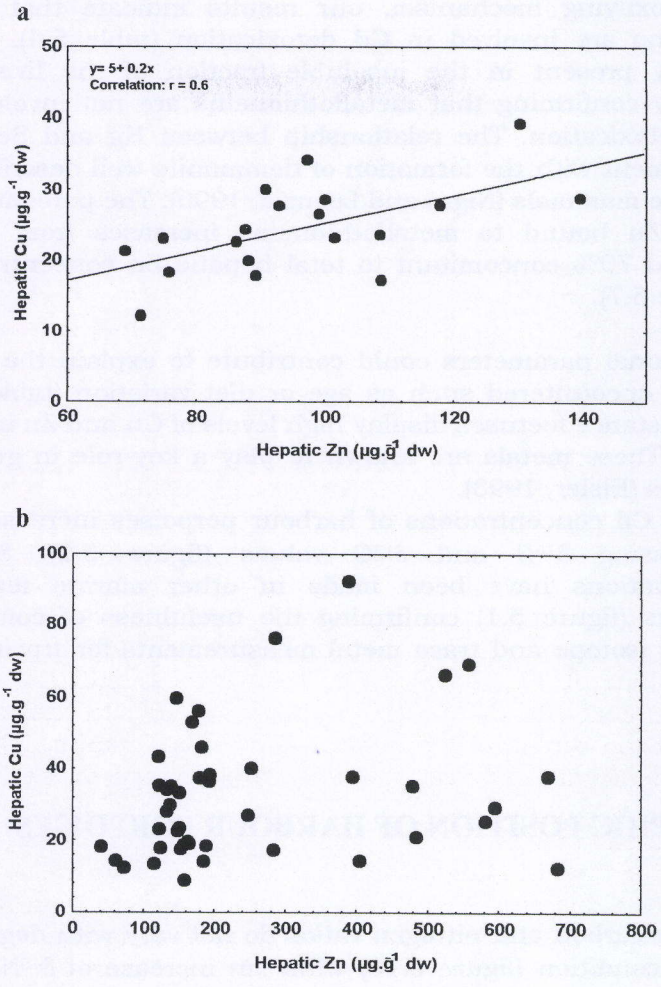

Figure 5.14. Relationship between hepatic Zn and Cu concentrations in porpoises caught accidentally in fishing nets (a, Norway) and found stranded along the Belgian coast (b)

Trace metals such as Zn or Hg could be particularly toxic for harbour porpoises and could well represent an additional source of stress to porpoises from the Southern North Sea already facing stressful condition such as elevated levels of organochlorines (Covaci et al., 2002).

When considering metallothioneins and their possible role as a detoxifying mechanism, our results indicate that these proteins are involved in Cd detoxication (table 5.4). Hg is mainly present in the insoluble fraction of the liver and kidney confirming that metallothioneins are not involved in Hg detoxication. The relationship between Hg and Se is in agreement with the formation of tiemmanite well described in marine mammals (Nigro and Leonzio, 1996). The percentage of total Zn bound to metallothioneins increases from 20 to around 70% concomitant to total hepatic Zn concentrations (figure 5.7).

Additional parameters could contribute to explain the metal levels encountered such as age or diet variation (table 5.5). For instance foetuses display high levels of Cu and Zn in their liver. These metals are known to play a key-role in growing tissues (Eisler, 1993).
Renal Cd concentrations of harbour porpoises increase with decreasing $\delta^{15}N$ and $\delta^{13}C$ values (figure 5.2). Similar observations have been made in other marine mammal species (figure 5.1) confirming the usefulness of combined stable isotope and trace metal measurements for tracing the diet.

TROPHIC POSITION OF HARBOUR PORPOISES

Stable carbon and nitrogen ratios do not vary with degrading body condition (figure 5.13) while an increase of $\delta^{15}N$ value has been reported for birds during experimental fasting (Hobson et al., 1993). Marine and hibernating mammals may possess biochemical pathways different from those of birds, in which no additional fractionation of nitrogen occurs (Ben-David et al., 1999). These results allow stable isotope comparisons between stranded and by-caught marine mammals. Porpoises from the German North Sea seem to feed at a higher trophic level than individuals from other locations (figure 5.9). Porpoises from the German Baltic Sea, Danish, and Belgian coasts display similar $\delta^{15}N$ values while $\delta^{13}C$ varies widely between locations. The $\delta^{15}N$ and $\delta^{13}C$ of marine predator tissues are determined initially by the isotopic

composition of the baseline phyto- and zooplankton sources. $\delta^{15}N$ and $\delta^{13}C$ values for Atlantic phytoplankton data may vary with their sampling origin. Trophic positions were estimated according to the model described by Lesage et al. (2001) for harbour porpoises from the Southern North Sea, German Baltic Sea and Norway coasts for which $\delta^{15}N$ values in the particulate organic matter (POM) were available (Middelburg and Nieuwenhuize, 1998; Velinsky and Fogel, 1999; Vos et al., 2000). A mean trophic position of 3.4 has been calculated for harbour porpoises from the Belgian part of the Southern North Sea. Assuming a similar $\delta^{15}N$ value around 9‰ for the German North Sea POM, porpoises from this area occupy a trophic position of 3.7 which is higher than animals from the Belgian coast. In contrast, porpoises from Norwegian coasts display a lower trophic position of 3.2.
The depletion in $\delta^{13}C$ observed for individuals from Norway and Iceland can be related to a more offshore feeding as the continental shelf is considerably reduced along the coasts of Norway and Iceland compared to the Southern North Sea bight. These results are enhanced by the high hepatic and renal Cd concentrations observed in porpoises from Norway and Iceland, suggesting a significant contribution of oceanic cephalopods to their diet.

CONCLUSIONS

Heavy metal absorption depends on a variety of factors often directly related to the metabolism and physiology of the animal. Marine mammals from the southern North Sea display strong intra- and inter-specific variations linked to the geographic location, the nutritional status, the age and the diet. As expected, by-caught animals from Norway and Iceland display a better nutritional status than stranded porpoises from the Southern North Sea as shown by their higher blubber thickness, total body mass to length ratio and hepatic to total body mass ratio. This may reflect the ability of healthy porpoises to mobilize fat from their blubber as a primary energy source and spare their protein reserves while porpoises in poor body condition depend primarily on muscle

protein catabolism. In this view, Zn, Hg, Se and Fe concentrations are clearly linked to emaciation, which in turn is linked to lesions of the respiratory system and parasitism as previously described (Siebert et al., 1999; Jauniaux et al., 2002). In addition, the constancy of liver mass during emaciation and the increase of hepatic Zn loads indicate a specific metabolism facing starvation, different from other mammals. Porpoises from the Southern North Sea (Belgian and German coasts) are carrying the highest burdens of Hg and Zn. In contrast, porpoises from Norwegian coasts display lower levels of these metals. These differences might be linked to both a difference in the anthropogenic impact of studied locations and nutritional status of the individuals. Excess of Zn and Hg could represent an additional source of stress to porpoises. Cd levels vary also greatly with locations with however no relation with the nutritional status. The relationship observed between Cd levels and stable isotopes for harbour porpoises and other species suggests that these variations are diet-related and that porpoises have a locally specific diet throughout their distribution area.

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CHAPTER 6: WHITE-SIDED DOLPHIN METALLOTHIONEINS: PURIFICATION, CHARACTERISATION AND POTENTIAL ROLE

After Das K., Jacob, V., Bouquegneau J.M. Comparative Biochemistry and Physiology Part C 131: 245-251

ABSTRACT

Metallothioneins (MTs) have been characterised in the kidneys of a white-sided dolphin *Lagenorhynchus acutus* stranded along the Belgian coast, displaying high levels of cadmium (Cd) and mercury (Hg) in liver and kidney. The protein has two isoforms: MT-1 and MT-2. MT-1 binds Cu, Zn, Hg and Cd while MT-2 only binds Zn, Hg and Cd. This suggest different metabolic functions for the two isoforms: MT-1 is mainly involved in Cu homeostasis; MT-2, which was four times more abundant than MT-1, detoxifies most of the accumulated cadmium.

6.1. INTRODUCTION

Since their first discovery by Margoshes and Vallee (1957), metallothioneins (MTs) have been detected studied in both prokaryotes and eukaryotes (Roesijadi, 1992; 1996) through more than 5000 research papers. MTs received their designation from their high metal and sulfur content. The mammalian forms are characterised by a low molecular mass of 6000-7000 Da, containing 60 to 68 amino acid residues, among which 20 cysteines. MTs bind a total of 7 equivalents of bivalent metal ions. However, the natural function of metallothioneins remains elusive. Previous studies on its structure, function, and molecular regulation have established a central role for these small proteins in the homeostatic regulation of essential metals including zinc (Zn) and copper (Cu) and detoxication of non-essential metals such as mercury (Hg) or cadmium (Cd) (Klaassen et al., 1999). In order to understand the functions of MTs, the functionality and structure of each MT isoform need to be considered. Indeed, in various mammal organs, such as liver or kidney, two major isoforms distinguishable by their charge, are present at low basal levels, MT-1 and MT-2 (Kägi, 1993). To date, however, no functional difference between the two isoforms has been firmly established (Moffat and Denizeau, 1997).
In the framework of heavy metal ecotoxicology, marine mammals appear as a choice material, since high levels of Hg and Cd can be naturally encountered in these animals (Das et al., 2001). The potential role of metallothioneins in marine mammals has been recently reviewed by Das et al. (2000). It appears that very few characterisations of marine mammal metallothioneins have been attempted. The first attempt was realised on sea lion, *Zalophus californianus*, and sperm whale kidney and liver, *Physeter macrocephalus* (Ridlington et al., 1981). Thereafter, MT I and II isolation and characterisation have been performed in the kidney of striped dolphin, *Stenella coeruleoalba* (Kwohn et al., 1986; 1988).

More recently, Pillet et al. (2000) have described the presence of both isoforms in the blood leucocytes of the grey seal, *Haliochoerus grypus*.

In order to obtain a better understanding on the role of metallothioneins in the detoxication and the dynamics of trace metals in marine mammals, detection, purification and trace metal content of white-sided-dolphin renal metallothioneins have been performed and compared with published data.

6.2 MATERIAL AND METHODS

The kidney and liver of an adult male white-sided dolphin *Lagenorhynchus acutus* found dead along the Belgian coasts on the 22nd September 1999 were sampled and stored at –20°C. These dolphin tissues were remarkably fresh (Jauniaux, personal communication) allowing protein analysis. The renal tissue contained 78% of water.

6.2.1. ACA 54 CHROMATOGRAPHY

3 to 4 grams of kidney were homogenized using an Ultra-Turrax in a 0.01 Mol ammonium formiate (pH = 7.4) containing 10 mM sodium azide and 0.01% dithithreitol and centrifuged at 26 000 g (60 min, 4°C). The supernatant was filtered on an AcA 54 gel column (1.6 X 64 cm) at 4°C. Fractions were collected and absorbance profile read at 215, 254 and 280 nm.

6.2.2. HEAVY METAL ANALYSIS AND MT CONCENTRATION ASSESSMENT

After adding nitric acid (65%) to each fraction and to the pellet, homogenate and supernatant, all were slowly heated to 100°C until complete digestion. Samples were diluted using deionised water and filtered prior to heavy metal analysis (Zn, Cu and Cd) by atomic absorption spectrophotometry (ICPS: ARL 3510). Hg was analysed by flameless atomic absorption (Perkin-Elmer MAS-50A) as described by Joiris et al. (1991). Quality control measurements for total mercury included replicate analysis resulting in coefficients of variation <10% and analysis of certified

material (DORM-1, NRC, Canada) as described by Debacker et al. (1997).
The Hg absolute detection limit is 10 ng corresponding to 0.13 $\mu g.g^{-1}$ fw for an average of 1.5 g fw. Detection limits for Cu, Cd and Zn were, respectively 0.18, 0.18 and 0.17 $\mu g.g^{-1}$. Quality of the analyses was controlled through participation in an intercalibration program (22). Recovery rates ranged from 94, 97, 100 and 102 % for Hg, Cu, Zn and Cd, respectively. Metal concentrations are expressed as $\mu g.g^{-1}$ fresh weight (fw.). From the heavy metal analyses, the MT concentration was estimated considering that Cu occurs in the divalent form and that 1 mole MT with a 6800 Da molecular mass typically binds 7 bivalent metal ions (7 gram-atoms per MT mole) (Kägi, 1993; Kojima et al., 1999).

6.2.3. DEAE SEPHADEX CHROMATOGRAPHY

Anion exchange chromatography was performed according to Kwohn et al. (1986). Briefly, after ACA 54 gel chromatography, the fractions with a low absorbance at 280 nm and a high absorbance at 254 nm were pooled and lyophilised (VIRTIS, 12XL). To eliminate the NaCl resulting from the lyophilisation, the proteins were chromatographed on a Sephadex G25 Medium column (1.5 x 28 cm). Metallothionein separation was performed on an anion-exchange Sephadex A25 (1.6 x 70 cm) column equilibrated with 1mM Tris-HCL buffer (pH 8.6). The column was eluted in a gradient mode with 1 to 250 mM Tris-HCl buffer, pH 8.6.
The ultraviolet spectra of renal MT-1 and MT-2 were recorded using the highest 0.2 nm resolution. DEAE Sephadex, Cd, Zn and Cu analyses, and UV spectra have been carried out in triplicate.

6.3. RESULTS

AcA-54 gel chromatography (figure 6.1.a) of the supernatant reveals a large band of metal (Zn, Cd, Cu) in the 10 kDa region of

the profile, consistent with the presence of metallothioneins. This metal peak is associated with a high absorbance at 254 nm and low absorbance at 280 nm related with the lack of aromatic amino acids of these proteins. This metallic peak shows a remarkably high temperature tolerance after a 3 minute heat treatment at 89°C followed by centrifugation (figure 6.1.b).

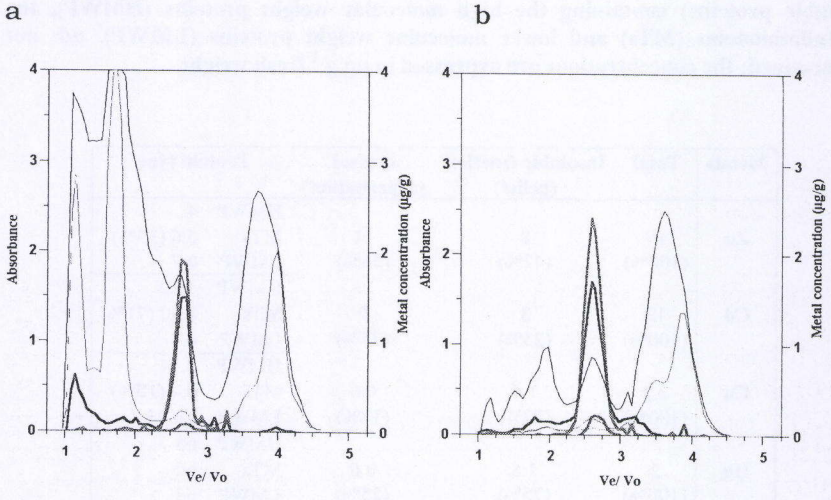

Figure 6.1. Elution profile on AcA 54 column of the water soluble fraction extracted from the kidney of the white-sided dolphin *Lagenorhynchus acutus* before (a) and after a 89°C heat treatment of the cytosolic fraction (b). The three types of bold lines represent the metal concentrations (-: Zn, -: Cd, -: Cu) of the fractions. The normal lines represent the protein absorption (-: 215, -: 250, 280 nm) Ve: elution volume; Vo: void volume of the column, mean volume per fraction: 2 ml

Table 6.1. shows the metal (Cd, Zn, Hg, Cu) distribution in the insoluble and soluble fractions including the metals bound on high molecular weight proteins, metallothionein-like proteins, and smaller molecules. High cadmium concentrations are found in the kidney (more than 12 $\mu g.g^{-1}$ fw) and liver (5 $\mu g.g^{-1}$ fw; data not shown). Almost 60% of the total Cd, 30% of the total Zn and 12% of the total copper are bound to cytosolic metallothioneins. The amount of metallothioneins in the kidney was calculated

from the metal content, assuming that the protein was fully saturated (7 equivalent of bivalent ions or 7 g-atoms per MT mole) with a molecular mass of 6800 kDa. A 172 $\mu g.g^{-1}$ fw concentration of metallothionein was calculated. After DEAE Sephadex A25 chromatography, the level of both isoforms estimated from trace metal data differ (figure 6.2.): MT-2 concentration was four times higher than that of MT-1.

Table 6.1. Zn, Cd, Cu, and Hg renal content in the insoluble fraction (pellet), cytosol (soluble proteins) containing the high molecular weight proteins (HMWP), the metallothioneins (MTs) and lower molecular weight proteins (LMWP). nd: not determined; the concentrations are expressed in $\mu g.g^{-1}$ fresh weight.

Metals	Total	Insoluble fraction (pellet)	cytosol (supernatant)	Protein type	
				HMWP	4
Zn	19	8	11	MTs	6.6 (35%)
	(100%)	(42%)	(58%)	LMWP	0.7
				HMWP	<0.2
Cd	12	3	9	MTs	8.30 (71%)
	(100%)	(25%)	(75%)	LMWP	0.3
				HMWP	0.14
Cu	2.2	1.6	0.6	MTs	0.4 (18%)
	(100%)	(72%)	(18%)	LMWP	<0.2
				HMWP	nd
Hg	2	1.5	0.6	MTs	nd
	(100%)	(75%)	(25%)	LMWP	nd

Figure 6.2 shows the elution profile of the DEAE Sephadex A-25 ion exchange chromatography. The profile reveals three metal containing fractions indicating that the MT was resolved into two isoproteins along the NaCl gradient. The first metal containing fraction (named I on the figure) did not bind to the column matrix and was eluted with 1mM Tris-HCl (pH 8.6) before the gradient started. This peak contains Zn, Cd, and Hg. The last two fractions were, in accordance with earlier experiments on mammalian liver, kidney and intestine, designated as MT-1 and MT-2 (Kwohn et al., 1986; Suzuki, 1991; Moffet and Denizeau, 1997). Hg concentrations were determined in pooled fractions (0.016 and 0.03 µg-atoms for MT-1 and MT-2 respectively).

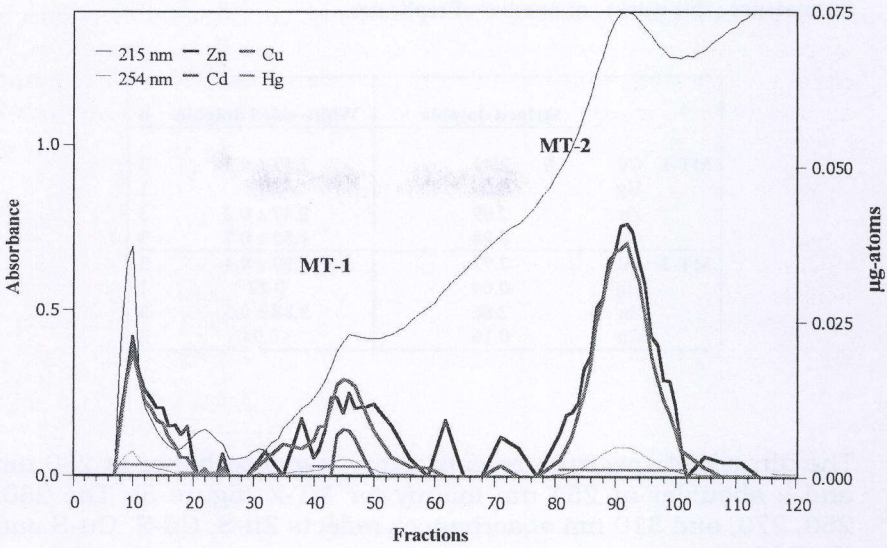

Figure 6.2. DEAE Sephadex A-25 chromatography profile of white-sided-dolphin metallothioneins.
Pooled and concentrated metallothionein fractions from AcA 54 column were applied to a DEAE Sephadex A-25 column. The three types of bold lines represent the Zn, Cd, and Cu concentrations of the fractions. Metal concentrations are expressed in µg-atoms (concentration expressed in $\mu g.g^{-1}$ divided by respective metal atomic mass). The horizontal dot lines represent the Hg concentration in the three pooled fractions. The normal lines represent the 215 and 250 nm protein absorption.

Furthermore, we observed that the metal composition of white-sided dolphin isometallothioneins varies in the different isoforms: MT-1 contains Zn, Cd, Hg and Cu while MT-2 contains Zn, Cd and Hg. Copper could not be detected in the second isoform (table 6.2).

Table 6.2. The molar ratio of metals to thionein (g-atoms/6000 g thionein) in striped (after Kwohn *et al.*, 1986) and white-sided dolphins (mean ± standard deviation of the measure ; this work) . n: number of replicates.

		Striped dolphin	White-sided dolphin	n
MT-1	Cd	2.41	2.57 ± 0.3	3
	Hg	0.06	0.39	1
	Zn	2.09	2.47 ± 0.2	3
	Cu	0.98	1.82 ± 0.7	3
MT-2	Cd	2.93	2.99 ± 0.4	3
	Hg	0.04	0.22	1
	Zn	2.86	3.88 ± 0.5	3
	Cu	0.16	<0.04	3

The ultraviolet spectrum reveals a very low absorbance at 280 nm and a shoulder at 254 nm mainly for MT-X (figure 3). The 230, 250, 270, and 310 nm absorbances reflects Zn-S, Cd-S, Cu-S and Hg-S binding.

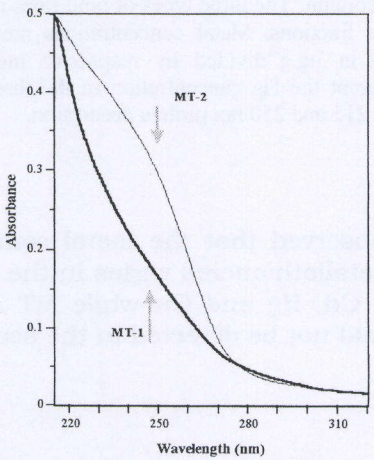

Figure 6.3: Ultraviolet spectrum of white-sided dolphin MT-1 and MT-2.

6.4. DISCUSSION

Cadmium is known to accumulate preferentially in the kidneys, bound in large amounts to MTs which also contain Zn and Cu. Renal and hepatic Cd concentrations measured in the male white-sided dolphin kidney are high (table 1). Indeed, the hepatic Cd concentration is ten times higher than the 0.5 $\mu g.g^{-1}$ (fw) measured previously in the liver of an adult white-sided dolphin from the U.S. Atlantic coast (Kuehl et al., 1994).

81% of the renal cadmium were found in the soluble fraction of the tissue bound mostly to low molecular weight soluble and heat-stable proteins (figure 6.1 and table 6.1) with high absorbance at 254 nm and lack of absorbance at 280 nm in agreement with MT specific features. The heat tolerance of these proteins is remarkable and implies that a single dosage of Cd, Zn, Cu and Hg in the heat-treated supernatant followed by centrifugation could allow the determination of the MT-bound metals and hence the renal MT concentrations. Complementary studies on other species are carried out in our laboratory to test the accuracy of such a method to quantify MTs in marine mammals.

Mercury is preferentially distributed in the insoluble fraction of the kidney (pellet, table 6.1), probably resulting from the well known formation of tiemannite (HgSe) in marine mammal livers and kidneys (Das et al., 2000; 2001).

Mammal kidney metallothioneins consist of two isoforms, MT-1 and MT-2 named in the order of their elution from anion-exchange column (Suzuki, 1991). The resolution of white-sided dolphin renal MT into two isoproteins (MT-1 and MT-2, Figure 6.2) on ion exchange chromatography corroborates the observations made on kidney and liver MT of monkey (Paliwal et al., 1982; 1986), human (Hellemans et al., 1999) or striped dolphin (Kwohn et al., 1986).
Cd, Zn and Hg bind to both isoforms while copper is not detected on MT-2. These results are consistent with a previous study on striped dolphin, *stenella coeruleoalba,* which has shown that MT-2 bind Cu in lower amount than MT-1 (Kwohn et al., 1986; table 6.2). These results seem to indicate a specific role of renal MT-1

in copper homeostasis, copper being an essential metal involved in many enzymatic processes (Eisler, 1997).
MT-2 is the major isoform present in the white-sided dolphin kidneys with a ratio MT-2/MT-1 concentration equal to 4.2. A ratio of 16 has been measured in striped dolphin kidney and 6.2 in equine kidneys (Kwohn et al., 1986). However, it is worth noticing that Wagemann *et* al.(1986) reported MT I as the major form of MTs in the liver and kidney from a narwhal. In fact, the relative abundance of each isoform can differ according to species, age, tissue and the nature of the inducers (Moffat and Denizeau, 1997). The higher MT-2 concentration probably results from a difference in the rate of synthesis of hepatic MT-1 and MT-2 which lead to a greater and more prolonged induction of MT-2 following administration of trace metals (Lehman-McKeeman et al., 1991) and a faster degradation rate of MT-1 compared to MT-2 (Mehra and Brenmer, 1985). As a whole, this leads to a more important role of MT-2 in the sequestration of Cd.

6.5. CONCLUSION

To conclude, white-sided dolphin metallothioneins display two renal isoforms MT-1 and MT-2, characterised by different metabolic functions in relation with copper cellular homeostasis (MT-1) and cadmium detoxification (MT-2). MTs appear to play a minor role in the binding and detoxification of Hg by marine mammals. Moreover, after appropriate heat treatment, trace metals measured in the supernatant are those bound to MTs, leading to a potential rapid and simple method to quantify these proteins in marine mammal tissues. Further experiments are needed to test the suitability of these method to others tissues and species.

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CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

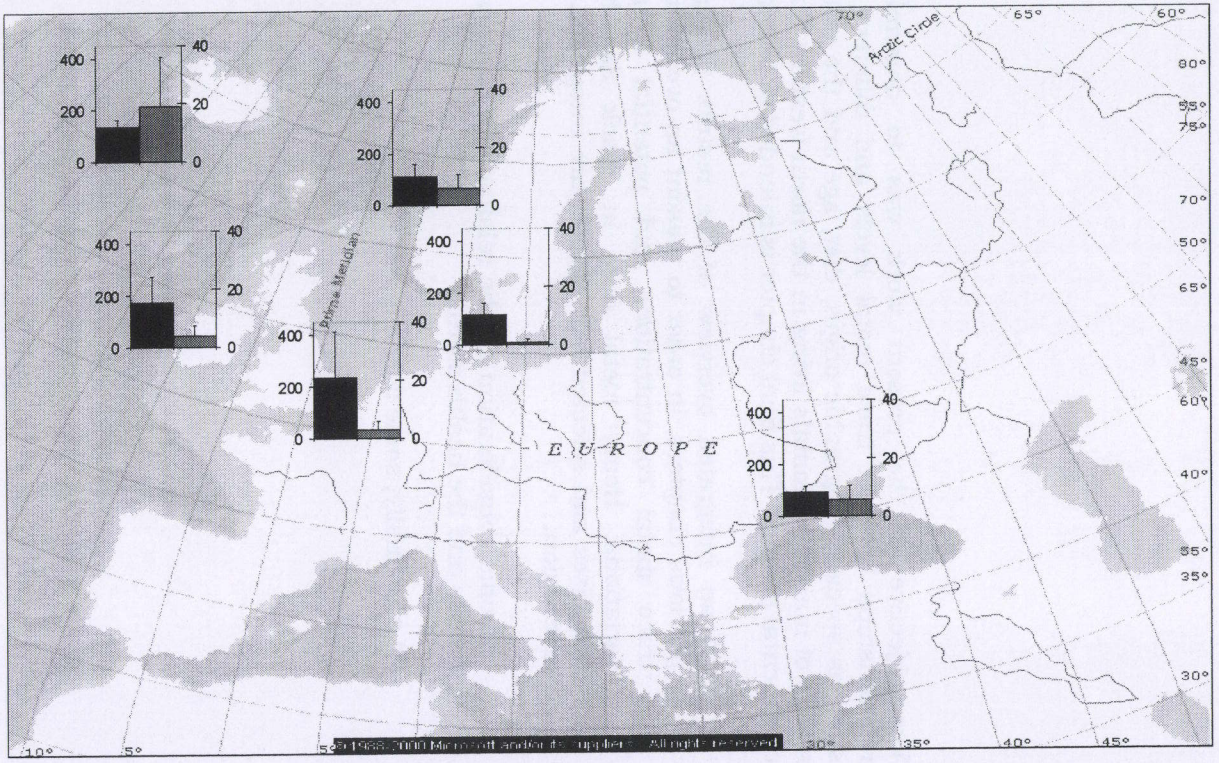
The objective of the present work was to determine the relationships between trace metal levels and different factors such as species, geographic origin, diet, age and the nutritional status of marine mammals living in the north-western European continental shelves. This final chapter is intended to provide:

(1) an integrated overview of the results presented in previous chapters, in order to present an overall image of trace metal concentrations and potential sources of variations. Heavy metal data in Black Sea harbour porpoises and selected fish species are integrated in this discussion.

(2) some consideration on the relationship between heavy metals and the health status

(3) and finally, some conclusions.

Map 7.1. Example of variations (mean ± sd) for hepatic Zn (blue bars) and renal Cd (red bars) in harbour porpoises



7.1. SOURCES OF VARIATIONS

During the present work, we have considered the sources of variation of trace metal concentrations in different marine mammal species from the North Sea and adjacent areas, as well as from the Black Sea (map 7.1): geographic origin, species, age, sex or body condition. Special attention has been given to the influence of diet using the stable isotope approach ($\delta^{13}C$ and $\delta^{15}N$).

7.1.1. THE DIET

Stable carbon isotopes ($^{13}C/^{12}C$) are clearly powerful tracers of the source of organic carbon sustaining communities, provided that the primary carbon sources are adequately characterized and differ in their $\delta^{13}C$ signatures. Stable isotopes of nitrogen ($^{15}N/^{14}N$) usually have little value as an indicator of the primary nitrogen source of a consummer's diet, but have been proven to be an indicator of the trophic level of an organism due to the more pronounced fractionation that occurs between trophic levels.

However drawbacks in its application must be mentioned: (1) the degree of fractionation shows rather large species variability, (2) the mechanisms underlying the fractionation of ^{15}N are still poorly understood. Therefore, when detailed information on the trophic position of a specific organism is required, it may be needed to first determine the actual degree of fractionation in laboratories or controlled field conditions (Hobson et al., 1996). Furthermore (3), baseline information on phytoplankton $\delta^{15}N$ is obviously needed to calculate a realistic predator's trophic position.

In many cases however, $\delta^{15}N$ data of consumers can still provide useful information. In our data set on Southern North Sea fish and invertebrates (chapter 4), for example, consistent differences in $\delta^{15}N$ values were clearly seen between higher and lower trophic level species or feeding groups.

Even more useful than the trophic status, $\delta^{15}N$ and $\delta^{13}C$ have provided a continuous data set in order to obtain a further understanding in the trace metal bioaccumulation and bioamplification processes (chapters 3 and 5).

For example, marine mammals display strong inter- and intra-specific Cd concentrations. Oceanic species such as sperm whales stranded on the Belgian coast (Holsbeek et al., 1999), striped and common dolphins from the Bay of Biscay, Irish and the French Channel coasts (chapters 2 and 3) displayed elevated renal Cd levels compared to harbour porpoises, grey and harbour seals and white-beaked dolphins (chapters 3 and 5). Unexpectedly, two adult white-sided dolphins stranded on the Belgian coast displayed very high Cd level (both 88$\mu g.g^{-1}$ dw).

Furthermore, harbour porpoises from the Icelandic coasts experienced more hepatic and renal Cd concentrations than porpoises collected along the Belgian coast (map 7.1, chapter 5). A significant relationship has been observed between both $\delta^{13}C$ and $\delta^{15}N$ values and Cd concentrations (liver and kidney) suggesting that part of this accumulation is diet related as a result of ingestion of prey experiencing high Cd level in their tissues and a depletion in both $\delta^{13}C$ and $\delta^{15}N$ compared to some marine mammals species. Such an isotopic signature and high Cd levels have been reported for various cephalopod species (Ostrom et al., 1993; Bustamante et al., 1998; Hooker et al., 2001).

No such obvious relationship has been observed between stable isotopes and other trace metals meaning that other factors are involved in the large variations observed for Zn, Hg, Se, Cu or Fe.

7.1.2. THE AGE

As expected, Cd and Hg accumulate strongly with age (or length where ages were not available) in mainly all analysed marine mammal species. Cu and Zn concentrations are also high in the liver of foetus in relation to their involvement in tissue development.

7.1.3. ENVIRONMENTAL CONTAMINATION

Zn and Hg concentrations measured in harbour porpoises stranded on the French Channel, Belgian and German North Sea coasts coasts were higher than those from Norway, Iceland and the Baltic sea. Part of the Zn and Hg variations are likely to be linked to the general body conditon of the harbour porpoises (see chapter 5 and paragraph 7.2. below) but these metal levels remain high even in animals displaying a good body condition. However metal data on by-caught porpoises from the Southern North Sea should help to confirm these results.

The Black Sea contamination deserves special attention, since environmental pollution of the surrounding mainland by heavy metals and organochlorines are expected to be high due to historical, agricultural and industrial habits, as well as limited water exchanges with the Mediterranean Sea (Mee, 1992). Annually the Danube alone discharges 60 tons of mercury and 6000 tons of zinc, which exceeds the entire river load to the North Sea (Mee, 1992). Surprisingly, hepatic Zn, Cu and Se encountered in by-caught harbour porpoises from the Ukraine were the lowest values of this study (map 7.1). Similar observations have been drawn for mercury, at about one order of magnitude lower than that in the North Sea (Joiris et al., 2001). Factors other than environmental contamination are likely to be involved: an abundant particulate matter within the North Sea on which metals get adsorbed (Martin et al., 1973 quoted by Dauby et al., 1994, Mart et al., 1982) and on the other hand the special hydrological conditions in the Black Sea with an important deeper anoxic zone. This might constitute an important sink of organic particulate matter (and metals adsorbed) and explain the low level of trace metals encountered in porpoises .

7.1.4. METALLOTHIONEINS INVOLVEMENT

Harbour porpoise metallothioneins represent 1.3% of the total protein concentrations. Despite this weak percentage, metallothioneins appear to have a key role in metal homeostasis as in the liver they bind 50% of the total Zn while only 19% is

present on the high molecular weight proteins (chapter 5). Metallothioneins are also involved in Cd detoxication as they bind 56% of the total renal Cd. Both in liver and kidney, metallothioneins appear to have a weak role in Hg detoxication as this metal is mainly distributed in the pellet, most probably associated with selenium as tiemmanite (HgSe). A significant relationship has been observed between total hepatic Zn and metallothioneins concentrations related to the Zn induction function in the cell.

Moreover, when Zn increases in the liver, the percentage bound to metallothioneins increases (from 20 to nearly 70%), suggesting that these proteins might take in charge the Zn overload resulting from the emaciation process.

Metallothioneins have also been characterised in the kidneys of a white-sided dolphin displaying high levels of cadmium and mercury in liver and kidney (see chapter 6). These preliminary results suggest that the protein has two isoforms: MT-1 and MT-2. MT-1 binds Cu, Zn, Hg and Cd while MT-2 only binds Zn, Hg and Cd. These two isoforms are likely to have different functions: MT-1 is mainly involved in Cu homeostasis; MT-2, which was four times more abundant than MT-1, detoxifies most of the accumulated cadmium.

7.2. TRACE METALS AND HEALTH STATUS

Trace metal levels were examined in relation to the general nutritional status of harbour porpoises described previously (figure 7.1.) (Siebert et al., 1999; 2001; Jauniaux et al., 2002). Other necropsy data such as lesions of the respiratory systems and total parasite burden were confronted to our toxicological results.

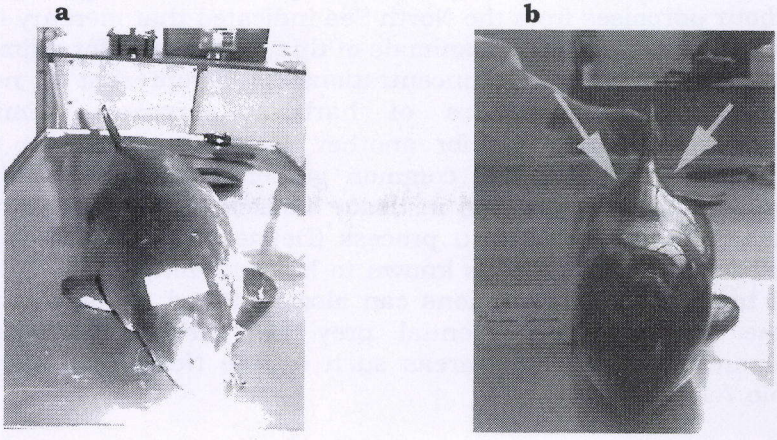


Figure 7.1. Harbour porpoises displaying a good (a) and emaciated (b) body condition (picture kindly provided by T. Jauniaux, ULG)

Previous studies mentioned higher metal levels associated with stranded or debilitated birds and marine mammals (Siebert et al., 1999; Debacker et al., 2001a,b, Bennet et al., 2001). The results of the present study further confirm these results and, more important, clearly point out a general rise of Zn and Hg in the liver of harbour porpoise as the emaciation worsens: the more the harbour porpoises get emaciated, the higher Zn and Hg levels in their tissue, while renal Fe decreases in relation to emaciation. Cu and Cd levels in tissues do not seem to be influenced by the body condition (chapter 5). In contrast to previous studies (Debacker et al., 2001a,b; Krämer et al., 1993), the increasing concentrations are not linked to the loss of the individual liver mass, which remains constant throughout the whole emaciation process. High Zn concentrations encountered in emaciated porpoises could be well related to a redistribution of zinc from other tissues such as muscle and so be a consequence of the emaciation process instead of a direct cause.

However, heavy metals (such as Zn and Hg) contamination may lead to a decrease of total body mass in various wildstock or laboratory animals (Ronald et al., 1977; Eisler et al., 1993; Debacker et al., 2001a,b). Zn also exerts a critical influence on the mammalian immune system (Salgueiro et al., 2000; Rink and Gabriel, 2001). Siebert et al. (1999) observed that harbour

porpoises from German North Sea are carrying a significant burden of mercury. The higher mercury content in organs from harbour porpoises from the North Sea indicated that mercury is a more important threat for animals of this region than for animals from the Baltic Sea. Hg concentrations could represent an non-specific morbidity factors of harbour porpoises. Similar conclusions were drawn for another species wintering in the Southern North Sea, the common guillemot *Uria aalge*. High heavy metal levels (Zn, Hg) in tissue associated to fasting could favor a general debilitation process (Debacker et al., 2001a,b). The Southern North Sea is known to be contaminated by metals and higher Zn concentrations can also be found in fish species (some of them are potential prey for harbour porpoises), compared to more open areas such as the Northeast Atlantic (table 7.1.)

Table 7.1. Muscle Zn and Hg (mean expressed in $\mu g.g^{-1}$ dw) in selected fish species from the North Sea and adjacent areas

fish species	Location	Zn	Hg	Reference
herring *Clupea harengus*	Southern North Sea	20	0.1	this work
	Northeast Scotland	6	0.05	Brown and Balls, 1997
	Northeast Atlantic	19	0.2	Cossa et al., 1990
cod *Gadus Morhua*	Central North Sea	3.6	0.18	Brown and Balls, 1997
	Southern North Sea	63	0.5	this work
sandeel *Ammodites sp*	Southern Nort Sea	207	0.1	this work
whiting *Merlangius merlangus*	Southern North Sea	55	0.1	this work
	Northeast Atlantic	17	0.4	Cossa et al., 1990
sole *Solea solea*	Southern North Sea	127	0.02	this work
	Northeast Atlantic	22	0.4	Cossa et al., 1990

Harbour porpoises are more likely to be susceptible to the contaminant potential detrimental effects compared to laboratory animals as they have to face a range of other natural and anthropogenic stressors such as capture in fishing nets and high levels of organochlorines (Covaci et al., 2002; Van de Vijver et al., submitted).

7.3. CONCLUSIONS

Within their Northeast Atlantic and Black Sea distribution area, marine mammals display strong intra- and interspecies variations in trace metal levels due to geographic origin, age, diet and trophic position but also by nutritional status of the individuals. For example, Zn and Hg concentrations are higher in harbour porpoises from the Southern North Sea compared to other areas. Some individuals are severely emaciated as shown by their muscle atrophy and reduced blubber thickness. Hepatic Zn, Se and Hg concentrations are significantly higher in emaciated porpoises than in normal animals.

It is generally assumed that animals exposed to high levels of metals for a long time have evolved mechanisms to minimize the potential detrimental effects of these contaminants. However, this does not exclude the possibility that the metals might affect the animals. Obviously, metallothioneins appear to have a key role in the homeostasis of Zn and Cu and in the detoxication of Cd. In contrast, Hg is mainly present in the insoluble fraction of the tissue in relation with the tiemmanite formation. The metallothioneins appear to be involved in the physiological response to the general heavy metal homeostasis disruption induced in the emaciated porpoises stranded along the Belgian coasts. The high Zn concentrations encountered in these animals are clearly linked to a general metallothionein synthesis. However the question rises and remains debatable to know at what cost debilitated individuals can still afford metallothioneins synthesis while consuming their protein reserves.

Although not at risk on a toxicological basis under normal conditions (robustness) the porpoises could well be adversely affected by Zn, Se and Hg with degrading body condition. Further investigations are needed before we can reach any definitive conclusions but we cannot reject the hypothesis that trace metal exposure may influence marine mammal health and contribute to the high mortality observed these last few years.

Apart from capture in fishing nets, which remains an important direct cause of mortality, marine mammals living near the coasts have to face various other stressful conditions such as trace metal and organochlorine contamination, habitat destruction, prey depletion and noise pollution. The Southern North Sea

obviously appears as an unfavourable environment for marine mammals as it combines all these detrimental stress factors and the question arises about the future and management of these species and their habitat.

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APPENDIX

Table A1: Heavy metal mean and range concentrations in livers (and kidney for Cd) from odontocetes: selected references. When the range was not available, standard deviation was used (all the data are expressed in dry weight assuming a mean water content of 75% of the tissues.

Species	Location	n (age estimation)	Hg-total	CH_3-Hg	Cd liver	Cd kidney	Se	Cu	Zn	Source
Pontoporia blainvillei	Argentina	2 (2and3y.)	**15**±6	nd	**13**±6	**40**±16	nd	**64**±9	**330**±160	Marcovecchio *et al.*, 1990
Platanista gangetica	India	4	nd	nd	**0.1** <0.04-0.15	**2** <0.04-6	nd	**207** 9-400	**126** 64-210	Kannan *et al.*, 1993
Tursiops truncatus	Argentina	1 (10y.)	**344**±29	nd	**3**±1	**114**±17	nd	**310**±1	**785**±136	Marcovecchio *et al.*, 1990
	South Carolina, USA	34	**71** <2-586	nd	**0.2** 0.02-1	nd	**38** 0.7-189	**43** 5-316	**227** 46-1084	Beck *et al.*, 1997
	Gulf of Mexico	10 Adult males	**180** 20-351	nd	**2** 0.4-5	nd	**74** 41-133	nd	nd	Kuehl and Haebler, 1995
Globicephala melas	Alaska	11	**180** 4-448	nd	nd	nd	**52** 6-114	nd	nd	
	Faroë Islands	Hg: n=8 Cd: n= 28	**852**±776	**17**±15%	**308** 6-668	**344** 6-976	nd 0-480	nd 8-60	nd 100-900	Caurant *et al.*, 1996 Caurant and Amiard-Triquet., 1995
	Newfoundland, Canada	26	**63** 0.3-298	nd	**78** 0-190	**43** 0-102	**31** 3-113	**17** 9-35	**264** 68-716	Muir *et al.*, 1988
Stenella coeruleoalba	Northeast Atlantic	Hg: n=8 Others: n=22	**206** 5-348	nd	**17** 0.2-51	**91** 0.1-199	nd	**43** 7-272	**167** 33-385	Das *et al.*, 2000 André *et al.*, 1991[b]
	Pacific Ocean, Japan	mature dolphins n=15	**820**±408	3.4%	nd	nd	**194**±115	nd	nd	Itano *et al.*, 1984
Delphinus delphis	Northeast Atlantic	28 Stranding	**128** 3-631	7%	**6** 0-96	**13** 0.4-81	**nd**	**12** 3-32	**143** 65-293	Holsbeek *et al.*, 1998
Lagenorhynchus albirostris	Newfoundland	26	**3** 0.5-6	nd	**2** 0.2-8	**14** 2-44	**8** 4-12	**20** 3-32	**100** 43-136	Muir *et al.*, 1988

Table A1 (continued).

Species	Location	n	Hg-total	CH_3-Hg	Cd liver	Cd kidney	Se	Cu	Zn	Source
Monodon monoceros	Baffin Island	38	**24**±12	/	**133**±13O	**298**±192	**16**±7	**21**±13	**151**±40	Wagemann *et al.*, 1983
	West Greenland	n>48	**21**[1] <0.02-171	/	**43**[1] <0.06-295	**156**[1] <0.06-500	**13**[1] <0.8-144	/	**144**[1] 53-271	Hansen *et al.*, 1990
Delphinapterus leucas	West Greenland	40	**7** 0.3-123	/	**9** <0.06-34	**41**	**15** 2-111	/	**114** 87-181	Hansen *et al.*, 1990
	St. Lawrence, Canada	30	**134** 1.5-808	/	**0.6** 0.004-2	**6** 0.004-15	/	/	:	Wagemann *et al.*, 1990
	Canadian western Arctic	77	**108** 1-464	/	**9** 0.5-27	**39** 15-88	**75** 3-235	**45** 3-140	**112** 43-185	Wagemann *et al.*, 1996
	Canadian eastern Arctic	73	**41** 5-154	/	**26** 0.03-103	**90** 0.3-314	**21** 1.5-91	**77** 3-1324	**115** 41-361	Wagemann *et al.*, 1996
Kogia breviceps	Argentina	1	47	/	30	1650	/	40	652	Marcovecchio *et al.*, 1990
Physeter macrocephalus	North Sea	6	**41** 9-61	**5%** 2-8%	**82** 52-175	**258** 133-426	**18** 6-43	**8** 5-12	**104** 90-125	Bouquegneau *et al.*, 1997[b] Holsbeek *et al.*, 1999
Phocoena phocoena	North Sea	5	**170** 1-504	37%	/	/	/	/	/	Joiris *et al.*, 1991
	Baltic sea	4	/	/	**0.3** 0.3-0.4	**1.5** 0.2-3	/	**24** 18-6	**120** 96-144	Szefer *et al.*, 1994
	West Greenland	44	**16** 2-80	/	**13** 0.2-45	**53** 0.4-290	**11** 2-36	**48** 20-200	**200** 145-370	Paludan-Müller *et al.*, 1993
Phocoenoides dalli	Nothwestern Pacific	3	6 (n=1; fœtus)	/	/ 0-84	/ 0-136	/	/ 20-329	/ 110-186	Fujise *et al.*, 1988

[1] Median

Table A2: Heavy metal mean and range concentrations in livers from mysticetes: selected references. Cadmium concentrations are also given for the kidney. All the data are expressed in dry weight, assuming a mean water content of 75% of the tissues. nd: not determined

Species	n	Location	Hg-total	CH_3-Hg	Cd liver	Cd kidney	Se	Cu	Zn	Source
Eschrichtius robustus	10	Western North America	**0.06** 0.01-0.1	nd	**4** 0.06-6	**4** 0.1-6	**2** 0.3-3	**9** 0.6-25	**99** 2-160	Varanasi *et al.*, 1994
Balaenoptera acutorostrata	135	Antarctic	**0.2** 0.1-0.5	nd	**38** 9-133	nd	nd	**17** 9-34	**146** 99-232	Honda *et al.*, 1986; 1987
	17	West Greenland	**2**[1] 1-11	nd	**4**[1] 2-6	**15**[1] 7-22	**6**[1] 4-10	nd	**138**[1] 106-192	Hansen *et al.*,1990
Balaenoptera physalus	11	Northeast Atlantic	**2** 0.6-5	40%	nd	nd	nd	nd	nd	Sanpera *et al.*, 1993
Balaena mysticetus	20	Alaska	**0.2** 0.08-0.4	nd	**31** 2-88	nd	**4** 1-9	**20** 12-40	**137** 88-261	Krone *et al.*, 1999

[1]: median

Table A3. Heavy metal mean and range concentrations in livers from pinnipeds: selected references. Cadmium concentrations are also given for the kidney. When the range was not available, standard deviation was used. All the data are expressed in dry weight, assuming a mean water content of 75% of the tissues.

Species	Location	n	Hg-total	CH_3-Hg	Cd liver	Cd kidney	Se	Cu	Zn	Source
Pusa hispida	Northwest Greenland	5	/	/	**232** 108-436	**1596** 1036-2324	/	/	/	Dietz et al., 1998
	Swedish coasts	4	**176** 19-348	/	**2.6** 0.6-3.4	**8** 5-22	**76** 11-112	**18** 15-19	**128** 72-180	Frank et al., 1992
Phoca vitulina	Swedish coasts	8	**104** 5-264	/	**0.4** 0.2-0.7	**2** 1-3	**44** 16-104	**34** 6-52	**216** 76-248	Frank et al., 1992
	Arctic	13	**24** 0.9-173	/	**12** 1-39	77 2-222	**14** 2-66	**26** 12-84	**171** 121-287	Wagemann, 1989
Halichoerus grypus	Swedish coasts	9	**104** 92-368	/	**0.7** 0.3-0.8	**5** 2-11	**56** 39-208	**56** 28-72	**184** 140-304	Frank et al., 1992
	Northwest England (Liverpool Bay)	12	**590** 6-1720	/	**2** <0.06-4	/	/	**53** 9-112	**218** 88-356	Law et al., 1992
Leptonychotes weddelli	Antarctica	3	**16** 0.2-34	/	**3** <0.02-5	**17** <0.02-40	/	**80** 76-103	**191** 166-220	Yamamoto et al., 1987

Table A3. Continued

Species	Location	n	Hg-total	CH_3-Hg	Cd liver	Cd kidney	Se	Cu	Zn	Source
Arctocephalus gazella	Georgia	11	**215** 52-334	/	**350** 55-684	/	/	**263** 132-438	**384** 259-643	Malcom et al., 1994
Arctocephalus philippii	Chile	**Pups** : n=27 **Adult** :n=1	**9** ± 6 **75**	/	**0.5** ± 0.2 **2**	/ /	/ /	/ /	/ /	Sepulveda et al., 1997
Odobenus rosmarus	Arctic	114	**4** 0.03-19	/	**38** 0.1-137	**244** O.1-564	**10** 2-20	**32** 6-137	**151** 50-300	Wagemann and Stewart, 1994